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THE PHARMACOLOGICAL EFFECTS OF ACUTE ETHANOL ON CATECHOLAMINES IN THE MEDIAL PREFRONTAL CORTEX AND DORSAL STRIATUM

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Dedication

To Don, Jenny, Christopher, and Dominic Vena for encouraging and supporting all of my academic and career achievements. To my husband, Nader for enduring this journey with me and keeping me sane throughout it.

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THE PHARMACOLOGICAL EFFECTS OF ACUTE ETHANOL ON CATECHOLAMINES IN THE MEDIAL PREFRONTAL CORTEX AND DORSAL STRIATUM

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The dorsal striatum and the medial prefrontal cortex are part of a neurocircuitry that is affected by acute and chronic drug use. In the present studies, we sought to characterize the pharmacological effects of ethanol on extracellular catecholamine concentrations in the dorsal striatum and medial prefrontal cortex. To this end, we utilized two different routes of administration to quantify ethanol's actions. We performed in vivo microdialysis in adult, male Long Evans rats as they received single or repeated intravenous infusions of ethanol. Following infusion of a 1-g/kg dose of ethanol, we observed no significant effects on extracellular dopamine in either the dorsomedial or dorsolateral striatum, but in a separate group of animals, we observed significant stimulation of extracellular norepinephrine in the medial prefrontal cortex. However, following a cumulative intravenous dosing protocol, we observed a gradual ramping up of tonic dopamine activity in the dorsal striatal subregions, which was more robust

in the dorsomedial striatum. Subsequently, we performed in vivo microdialysis in separate groups of rats during an operant self-administration session to quantify the time course of extracellular dopamine and norepinephrine in the medial prefrontal cortex. In the seven operant sessions prior to the microdialysis test session, each group of rats had been assigned to a separate treatment group: one that received a sweetened ethanol solution, one that received a sucrose solution, and a handling control group that did not receive any drinking solutions. In the ethanol-experienced animals, we report a reduction in basal dopamine and norepinephrine in the medial prefrontal cortex, relative to control groups. However, there were no significant differences in the temporal profile of extracellular norepinephrine across the three treatment groups. These studies demonstrate that limited voluntary ethanol consumption appears to be sufficient to alter tonic catecholamine signaling in the medial prefrontal cortex. Additionally, we conclude that central catecholamine signaling pathways are a target for ethanol.

Table of Contents

List of Tables

List of Figures

Chapter 1:
Background

ALCOHOL USE DISORDERS

Definition and global impact

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines alcohol use disorders (AUDs) as a condition in which "a patient's drinking causes distress or harm" (NIAAA, 2016). The most recent version of the Data and Statistics Manual of Mental Disorders V (DSM-5; American Psychiatric Association, 2013) utilizes AUD in lieu of two previously distinct conditions – alcohol abuse and alcohol dependence. Similar to other substance use disorders, core symptoms of AUDs include the compulsive drug seeking and consummatory behaviors even in the face of negative consequences, loss of control over alcohol use, and the development of a negative affective state when alcohol use is discontinued (Koob & Volkow, 2016).

In 2014, 88% of adults (18 years or older) in the U.S. reported that they had used alcohol in their lifetime, and 57% reported using alcohol in the past month, a criterion commonly used to identify regular use (NIAAA, 2016). While many individuals will engage in problematic drinking behaviors, such as binge

and heavy drinking, few go on to develop severe alcohol use disorders, or alcoholism (NIAAA, 2016). In 2014, about 7% of adults in the U.S. had an AUD, with rates twice as high for men as women.

The global economic and societal burdens of alcohol abuse are disproportionately staggering relative to other conditions that have greater prevalence. In the U.S. alone, the costs associated with alcohol misuse in 2010 we $249.0 billion (NIAAA, 2016). According to the World Health Organization Global Status Report on Alcohol and Health (2014), 5.1% of the global burden of disease and injury was due to alcohol consumption.

These statistics highlight the need for research into the development and expression of alcohol use disorders. Understanding the biological, psychological, and neurological components of this condition will be critical to the development of preventative and early intervention measures, improvement of the current standard of care and treatment, and to influencing public policy and awareness.

Proposed neurobiology

A dramatic shift occurs in the motivation to seek and use drugs, including alcohol that precedes the development of maladaptive and pathological substance use. Initially, drug-seeking behavior is goal-directed and may be impulsive, mediated by the positive reinforcing effects of the drug (Koob & Volkow, 2016; Mangieri, Cofresí, & Gonzales, 2012). With alcohol, such positive

hedonic features may include anxiolysis, euphoria, and increased sociability. Following repeated cycles of intoxication, withdrawal, and craving, drug-seeking behaviors become more automated and habitual. As the motivation to seek drugs becomes an attempt to resolve the negative affective state induced by withdrawal, substance use behaviors become compulsive, driven by an urge to resist tension or anxiety (Volkow, Koob, & McLellan, 2016).

Currently, the proposed neurobiological mechanisms underlying the progression from impulsive to compulsive drug use exists within a conceptual framework of drug dependence as established by Dr. George Koob and colleagues. This framework consists of three primary domains: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation. Each of these domains is associated with adaptations in specific circuits. For example, a common feature of all abused drugs is their ability to acutely stimulate the mesocorticolimbic dopamine system, which contributes to the positive reinforcing effects of drugs and alcohol. This central circuit consists of dopamine neurons originating in the ventral tegmental area (VTA) and projecting to the prefrontal cortex, nucleus accumbens, dorsomedial striatum, ventral pallidum, and other regions (Everitt & Robbins, 2005; Koob & Volkow, 2010). Repeated stimulation of this circuit by exogenous rewards facilitates the learning of stimuli that are associated with the reward (Schultz, 1997). Over time neuroadaptations within this circuit may promote enhanced motivation for drug-related stimuli over natural reinforcers.

Following a binge/intoxication experience, an individual may experience unpleasant and potentially stressful effects that occur when the drug is eliminated from the body. The emergence of withdrawal symptoms, such as dysphoria, pain, and malaise, is one of the criteria listed in the DSM-5 for diagnosing substance use disorders (American Psychiatric Association, 2013). The withdrawal/negative affect domain is characterized by increases in reward thresholds, an escalation in drug consumption, and activation of stress and anxiety circuits within the extended amygdala, habenula, ventral tegmental area, and the nucleus accumbens (Koob & Volkow, 2016). The aversive and negative affective state induced by acute or protracted abstinence can be alleviated by consumption of the drug, which contributes to the development of craving and appetitive behaviors, and eventually may progress to habitual and compulsive drug seeking behavior (Koob & Volkow, 2016).

Chronic drug use is associated with deficits in executive functions, which are primarily governed by the prefrontal cortex. Impairments in decision-making, inhibitory control, working memory, and behavioral flexibility are believed to be the result of neuroplastic changes in dopamine and glutamatergic signaling observed in the frontal cortex of humans and animals following long-term chronic drug use (Koob & Volkow, 2016; Volkow et al., 2016). Neuroadaptations within the frontal cortex may also contribute to the development of incentive salience and the incubation of craving by drug-associated stimuli, which are characteristics of the preoccupation/anticipation domain. The ability of drug-

associated cues to activate reward and emotional/motivational circuitry is a critical factor in the development of habitual and compulsive drug seeking behaviors.

THE DORSAL STRIATUM

Anatomy

The dorsal striatum, or the caudate-putamen in primates, is the largest component of the basal ganglia (Gerfen & Surmeier, 2011). The basal ganglia connect essentially all cortical areas involved in the planning and executive control of actions with regions that modulate behavior (Gerfen, 2004; Gerfen & Surmeier, 2011). The major inputs to the basal ganglia are glutamatergic neurons of the neocortex and midbrain dopamine neurons, which target the dorsal striatum.

The dorsal striatum integrates sensorimotor, motivational and emotional information to direct an appropriate behavioral response. GABAergic medium spiny neurons originating in the dorsal striatum are the major output neurons of the basal ganglia and can be divided into two distinct populations based on their projections. One population directly innervates the GABAergic neurons in the medial globus pallidus and SN pars reticulata, which target thalamic nuclei that coordinate movement generation (Gerfen, 2004). This “direct” projection system is characterized by the presence of D1 dopamine receptors and appears to

generally excite downstream neural systems that affect behavior (Gerfen, 2004; Gerfen & Surmeier, 2011; Yin & Knowlton, 2006). In contrast, the “indirect” projection system innervates an intermediate nucleus, the lateral globus pallidus, which together with the subthalamic nucleus, targets behavior output systems within the thalamus (Gerfen, 2004; Gerfen & Surmeier, 2011). This indirect pathway is characterized by the presence of D2 dopamine receptors on medium spiny neurons and appears to generally inhibit circuits regulating behavioral output and motor activity. Therefore, striatal output is critically modulated by dopamine activity. Dopamine neurons projecting to the dorsal striatum originate in ventral tegmental area and substantia nigra pars compacta, and are highly sensitive to action outcomes.

Habitual behavior definition

As discussed above, drugs of abuse, including alcohol, activate the mesocorticolimbic dopamine pathway, which is associated with the stimulating and reinforcing aspects of the drug itself (Di Chiara & Imperato, 1988; Yin, Ostlund, & Balleine, 2008). This may contribute to the motivation to voluntarily seek and consume drugs. Such behavior is considered goal-directed because it is sensitive to the value of the outcome, and may be modified based on the perceived changes in the value of the outcome. With extended training and experience, the behavior becomes automated or habitual, and is not altered in

response to reward devaluation (Corbit, Nie, & Janak, 2012; Faure, Haberland, Condé, & El Massioui, 2005; Jonkman, Pelloux, & Everitt, 2012; Yin, Knowlton, & Balleine, 2004). Rather than performing an action to attain a specific outcome, a conditioned stimulus automatically elicits a response, and this may be the precursor to compulsive behavior. As evidenced by animal models of drug use, with prolonged training drug-associated sensory cues acquire incentive salience and as conditioned reinforcers, they can drive drug-seeking behavior on their own (Dickinson, Wood, & Smith, 2002). In humans, a stimulus may be a context in which drug use has previously occurred, an internal emotional state (i.e. stress/anxiety), a specific object that is associated with the drug, or the drug itself.

The dorsal striatum, dopamine, and habit formation

The current literature suggests that the shift in drug-seeking behavior from voluntary to habitual, observed after chronic and prolonged use of a drug, may be due to a transition in the neuroanatomical substrates controlling behavior, possibly via a dopamine-dependent mechanism (Corbit et al., 2012; Everitt & Robbins, 2013; Volkow, Wang, Fowler, Tomasi, & Telang, 2011; Yin & Knowlton, 2006). Acute drug administration is associated with increased dopamine activity in the ventral striatum, a region necessary for the acquisition of goal-directed behavior and instrumental conditioning (learning the association between an

action and the presentation of a reward) (Everitt & Robbins, 2013). However, if drug seeking and consumption behavior is maintained over an extended period of time, there appears to be recruitment of the dorsal striatal dopaminergic circuit, as indicated by human and animal studies (Balleine, Delgado, & Hikosaka, 2007; Volkow et al., 2011; Willuhn, Burgeno, Everitt, & Phillips, 2012). Actions that produce positive hedonic outcomes stimulate dopamine release into the striatum, which is critical for synaptic plasticity and reinforcement learning. This may enable drug-associated stimuli to assume a greater influence in predicting the expression of appetitive behaviors (Everitt & Robbins, 2013).

The dorsal striatum has functionally distinct lateral and medial subregions that display differential neuronal firing patterns in response to ethanol and ethanol-associated cues (Fanelli, Klein, Reese, & Robinson, 2013). These subregions appear to have different roles in the regulation of behavior, with the dorsomedial striatum (DMS) regulating goal-directed behavior and the dorsolateral striatum (DLS) regulating automated stimulus-response behaviors (Balleine et al., 2007; Everitt & Robbins, 2013; Faure et al., 2005; O'Doherty et al., 2004; Yin & Knowlton, 2006; Yin et al., 2004, 2008). Corbit et al. (2012) demonstrated the dichotomous roles of the DMS and DLS using an operant model of alcohol self-administration in rats. Following limited alcohol experience (after 2 weeks of operant self-administration), responding for alcohol is sensitive to devaluation, an effect that is blocked by inactivating the DMS with GABA agonists. After prolonged alcohol exposure (after 8 weeks of operant self-

administration), responding for alcohol is insensitive to reward devaluation, indicating that control over behavior has shifted to a habit-based mechanism. Inactivating the DLS reversed this effect. Other manipulations of the dorsal striatal subregions, including lesion studies and studies testing other drugs of abuse, support these findings (Yin et al., 2004; Yin, Ostlund, Knowlton, & Balleine, 2005).

It is generally supported that dopamine signaling in the dorsal striatum is critical for the reinforcement learning that underlies the development of habitual drug seeking behavior (Belin & Everitt, 2008; Centonze, Picconi, Gubellini, Bernardi, & Calabresi, 2001; Faure et al., 2005; Ito, Dalley, Robbins, & Everitt, 2002; Kreitzer & Malenka, 2005; Lovinger, 2010). Furthermore, ethanol has been shown to enhance plasticity among D1-containing medium spiny neurons (Wang et al., 2015). However, few studies have looked at the direct pharmacological effects of ethanol on dopamine in the dorsal striatum. Quantitating these effects, particularly within each of the dorsal striatal subregions, is necessary for understanding dopamine's role in the development of habitual alcohol seeking behaviors.

THE PREFRONTAL CORTEX

Anatomy

The prefrontal cortex (PFC) is a large heterogeneous region located at the anterior pole of the frontal cortex (Abernathy et al, 2010). The PFC is extensively connected with sensory systems, cortical and subcortical motor coordination systems, autonomic systems, and limbic and midbrain regions involved with motivation, reward, emotion, and memory (Miller & Cohen, 2001; Ongür & Price, 2000). Therefore, the PFC is well-situated to exert top-down control over behavior. It consists of 3 interconnected subregions: lateral PFC, medial PFC, and orbital PFC. Because the studies described herein analyzed the effects of ethanol on neurochemistry within the medial PFC (mPFC) in rodents, discussion of all PFC subregions is beyond the scope of this manuscript.

Within the mPFC, about 80-90% of the neuronal population is excitatory pyramidal cells, while the remaining 10-20% is GABAergic interneurons (Riga, Matos, et al., 2014). The mPFC in rodents can be subdivided into the prelimbic mPFC (functionally analogous to the dorsolateral PFC in humans and primates) and the infralimbic mPFC (functionally analogous to the ventromedial PFC in humans and primates), which are reciprocally connected (Abernathy, Chandler, & Woodward, 2010; Riga, Matos, et al., 2014; Vertes, 2006). The mPFC strongly projects to cortical and subcortical structures involved in motor coordination (Abernathy et al., 2010; Miller & Cohen, 2001). The prelimbic region provides

glutamatergic projections to the nucleus accumbens core and the dorsomedial striatum, while the infralimbic region densely innervates the nucleus accumbens shell (Abernathy et al., 2010; Ongür & Price, 2000; Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). These circuits are likely involved in reward-guided choice behaviors. Also implicated in reward (and aversion) processing are the reciprocal projections between the mPFC and the ventral tegmental area, which is also innervated by noradrenergic neurons originating in the locus coeruleus. Cortical processing of internal affective states and memory appears to occur via reciprocal projections between the mPFC and the hippocampus and between the mPFC and the amygdala (Abernathy et al, 2010; Ongür & Price, 2000).

mPFC functional roles

With the evolution of more complex organisms, it became necessary for the development of a brain region to mediate higher-order executive functions such as reasoning, attention, planning, working memory, and decision making (Abernathy et al, 2010; Riga et al, 2014). To ensure survival, consistent monitoring of internal goals and external environmental patterns became necessary, as well as the ability to flexibly orchestrate complex behavioral responses. The current literature indicates that these roles appear to be dependent on the mPFC (Miller & Cohen, 2001). To accomplish this, the mPFC

must attend to relevant stimuli, suppress responding to irrelevant stimuli, plan behavioral sequences based on environmental stimuli and past experiences, and select appropriate motor behaviors in response to environmental stimuli and internal states (Miller & Cohen, 2001; Abernathy et al, 2010).

Attention and behavioral flexibility

Recent work implicates the mPFC in specific aspects of attention, learning, and behavioral flexibility. For example, inactivation or lesions of the mPFC do not impair acquisition of visual cue discrimination learning or response discrimination learning, nor do such manipulations impair reversal learning, but they do impair extradimensional set-shifting behaviors (Birrell & Brown, 2000; Floresco, Block, & Tse, 2008; Killcross & Coutureau, 2003). Extradimensional set-shifting behaviors require an organism to reorient attention to a different set of sensory features of complex stimuli, while discrimination or reversal learning does not require shifting attention to previously irrelevant stimuli (Birrell & Brown, 2000; Dalley, Cardinal, & Robbins, 2004). Another task for assaying directed and sustained attention, is the 5-choice serial reaction time task (5-CSRTT). Damage to the mPFC produces substantial deficits in accuracy on this task, and is associated with premature and perseverative responding (Chudasama et al., 2003; Robbins, 2002). These studies and others support a general role for the mPFC as a supervisory attentional and behavioral control center (Perry et al.,

2011). Supporting these preclinical findings is a study in humans with surgical lesions of the dorsolateral PFC. While some of these patients performed successfully on a visual discrimination reversal task, those that showed impaired performance reported deficits in attentional control (Hornak et al., 2004). In summary, these studies support the role of the mPFC in monitoring and flexibly attending to motivational and contextual stimuli to guide decision-making and adaptive behaviors.

Regulation of appetitive instrumental behavior

The ability to detect and respond to contextual stimuli predicting risk or reward is critical for survival. The PFC is central to reward-based decision-making, but it is functionally heterogeneous in processing specific aspects of reward seeking and acquisition (Tzschentke, 2000). The medial PFC appears particularly important in orienting attention and arousal towards reward-predicting stimuli to drive appetitive behaviors. Additionally, evidence suggests that the mPFC may be necessary for the reinforcing effects of some drugs of abuse. For example, mPFC lesions prevent the acquisition of conditioned place preference (a behavioral assessment of the subjective rewarding aspects of reinforcers) for morphine and cocaine (Hao et al., 2008; Tzschentke, 2000). Animals will also self-administer cocaine into the mPFC (Goeders & Smith, 1983).

Because the mPFC regulates working memory, it is central in tracking temporal and discriminative information related to reward-associated rules and cues. In reinforcement learning, action selection is partly determined by the outcomes of previous choices. Indeed, work has shown that neural signaling in the mPFC increases during the onset of a chosen action (Matsumoto, Suzuki, & Tanaka, 2003), and remains elevated until its outcome (Sul, Kim, Huh, Lee, & Jung, 2010). Upon receipt of a rewarding outcome, neural signaling was further enhanced (Sul et al., 2010). Persistent neural activity observed in the mPFC (or dlPFC of primates) during a delay period, either between the behavioral response and the outcome delivery or between trials, is believed to be a correlate of temporary active maintenance of information relating an action choice on a given trial to the outcome of the trial (Bouret & Sara, 2004; Funahashi, 2006; Matsumoto et al, 2003; Sul et al., 2010). Interestingly, this pattern of sustained neural activity is only observed when the behavioral sequence is performed in the appropriate context (Mulder, Nordquist, Örgüt, & Pennartz, 2003). Therefore, the mPFC appears to regulate goal-directed responding triggered by stimuli that predict an anticipated outcome.

Consistent with this hypothesis, the mPFC has been implicated in cue-induced reward seeking behavior. Drug-related contextual cues and cue-induced drug seeking are associated with neuronal activation in the mPFC of rats (Ball & Slane, 2012; Bossert et al., 2012; Koya et al., 2009). For example, tetrodotoxin injections into the mPFC attenuated the ability of a cocaine-paired context to

reinstate extinguished cocaine seeking behavior (Fuchs et al., 2005). In humans, increased glucose metabolism has been observed in the dorsolateral PFC in cocaine abusers upon presentation of drug-related stimuli (Grant et al., 1996). Mechanistic studies have implicated prefrontal cortical glutamateric and dopaminergic signaling in conditioned appetitive behaviors (Baldwin, Sadeghian, & Kelley, 2002), though it is likely that other systems are involved. For example, downregulation of glutamatergic transmission in the mPFC is associated with cue-induced heroin reinstatement in rats (Van den Oever et al., 2008; Van den Oever, Spijker, Smit, & De Vries, 2010).

Other work has attempted to delineate specific roles of the prelimbic and infralimbic subregions in appetitive behaviors. Inactivation of the dorsal mPFC (prelimbic PFC) inhibited cue-induced sucrose seeking behavior. In contrast, inactivation of the ventral mPFC (infralimbic PFC) appeared to increase responding to unrewarded cues, an indication of reduced inhibitory control (Ishikawa, Ambroggi, Nicola, & Fields, 2008). In rats that had undergone extensive extinction training for cocaine self-administration, inactivation of the infralimbic PFC induced reinstatement of cocaine seeking, while activation of the infralimbic PFC suppressed cocaine-induced reinstatement (Peters, LaLumiere, & Kalivas, 2008). Together, these data suggest a specific role of the infralimbic cortex in inhibitory control over conditioned behavior. However, other work has yielded inconsistent results. For example, rats with prelimbic PFC lesions showed increased premature responding and shortened response latencies in a

cue-triggered reaction time task (Narayanan, Horst, & Laubach, 2006). Another study demonstrated abolishment of context-potentiated food consumption in sated rats with lesions of the infralimbic cortex relative to sham-lesioned controls (Petrovich, Ross, Holland, & Gallagher, 2007). The use of different behavioral tasks may explain the inconsistent results from these lesion/inactivation studies, but further investigation is necessary to determine the precise functional roles of the prelimbic and infralimbic cortices in inhibitory control. Nevertheless, the studies discussed above implicate the mPFC in the regulation of reward-seeking behaviors in response to environmental stimuli.

Role of catecholamines in mPFC functions

The catecholamines dopamine and norepinephrine are critical neuromodulators in various mPFC-mediated functions, including working memory, arousal and attention, behavioral flexibility, and stress- and reward-related behaviors (Dalley et al., 2004; Jett & Morilak, 2013; Lapiz & Morilak, 2006; Riga, Matos, et al., 2014; Zvani L Rossetti & Carboni, 2005). However, it has been difficult to accurately delineate the specific roles of cortical dopamine and norepinephrine in behavior (Feenstra, 2000). One observation that has been consistently reported is that catecholamines can exert a biphasic effect on cognitive and executive functions, following an inverted-U shape concentration-response curve (Arnsten, 1997; Cools & D'Esposito, 2011; Dalley et al., 2004).

Because basal catecholamine concentrations in the mPFC are relatively low, small fluctuations in extracellular concentrations can have significant effects on mPFC-mediated functions. Tonic catecholamine levels are typically low in fatigued/low arousal states, and increase in response to stress or in cases of heightened vigilance (Aston-Jones, Rajkowski, & Cohen, 1999; Jett & Morilak, 2013; Nakane, Shimizu, & Hori, 1994). Reduced extracellular catecholamine signaling can result in increased distractibility and poor impulse control, which will hinder appropriate responding in cognitively taxing situations (Arnsten & Pliszka, 2011; Carli, Robbins, Evenden, & Everitt, 1983; Cole & Robbins, 1992). Moderately elevated catecholamine levels (such as in an alert, nonstressed state) can improve behavioral flexibility, attention to salient stimuli, and working memory, which are necessary in the face of environmental challenges. This engagement of the mPFC appears to be mediated via dopamine D1 receptors and high-affinity α2 adrenergic receptors, as determined by pharmacological studies (Arnsten & Pliszka, 2011; Riga et al., 2014; Robbins, 2002; Spencer, Devilbiss, & Berridge, 2015; Wang et al., 2007). In contrast, excessive activation of dopamine D1 receptors (Arnsten, 1997; Rios Valentim, Gontijo, Peres, Rodrigues, & Nakamura-Palacios, 2009; Robbins, 2002; Vijayraghavan, Wang, Birnbaum, Williams, & Arnsten, 2007) and α1 adrenergic receptors (Lapiz & Morilak, 2006; Ramos & Arnsten, 2007) will impair mPFC functioning, as is the case following exposure to stress or high doses of stimulant drugs. Chronic stimulation of these receptors can result in an inability to distinguish between

relevant and irrelevant stimuli and impair response accuracy in attentional tasks (Blaine, Milivojevic, Fox, & Sinha, 2016).

It has been difficult to characterize precise roles for dopamine and norepinephrine in the mPFC for several reasons, a few being: (1) extracellular concentrations of dopamine and norepinephrine are regulated by overlapping mechanisms (Morón, Brockington, Wise, Rocha, & Hope, 2002; Pan, Yang, & Lin, 2004; Tanda, Pontieri, Frau, & Di Chiara, 1997), (2) the mPFC is especially sensitive to small changes in neurochemical activity (Arnsten & Pliszka, 2011; Ramos & Arnsten, 2007), (3) cortical dopamine and norepinephrine activity increases in response to salient stimuli, regardless of valence (Feenstra, 2000; Popescu, Zhou, & Poo, 2016), and (4) recent work has provided evidence of the co-release of dopamine and norepinephrine from noradrenergic terminals (Devoto, Flore, Pani, & Gessa, 2001; Devoto, Flore, Pira, Diana, & Gessa, 2002; Devoto, Flore, Saba, Fà, & Gessa, 2005). In spite of these confounds, however, some work has suggested differential roles of cortical dopamine and norepinephrine in specific aspects of behavior. During performance on a T-maze task of spatial working memory, Rossetti and Carboni (2005) observed increases in extracellular concentrations of both catecholamines during the task. Interestingly, they observed significantly greater increases in noradrenergic activity in rats trained to alternate entrances into the left and right arms on the maze with each trial, as compared to untrained animals. This finding suggests specific recruitment of mPFC noradrenergic activity in more cognitively

demanding situations, possibly because such situations require heightened arousal and selective orientation of attention (Rossetti & Carboni, 2005; Robbins, 2002).

The same study also reported increased dopamine, but not norepinephrine concentrations during the waiting/anticipatory period prior to the start of the task, which concluded with the animal obtaining food reinforcement. This is consistent with the role of mesocortical dopamine in reward prediction error and reward expectancy (Jo & Mizumori, 2016; Merali, McIntosh, & Anisman, 2004; Parker, Alberico, Miller, & Narayanan, 2013; Rossetti & Carboni, 2005). An additional role of cortical dopamine appears to be in enhancing the signal to noise ratio of relevant stimuli by reducing mPFC responsiveness to irrelevant stimuli (Arnsten & Pliszka, 2011; Berridge & Devilbiss, 2011). Together, these studies indicate that while dopamine and norepinephrine dually modulate many mPFC functions, these systems may differentially modulate precise aspects of cognitive behaviors, but this hypothesis requires further investigation.

Appetitive behaviors

The role of mesocortical dopamine in appetitive behaviors has been the subject of much research, but the role of norepinephrine has received considerably less attention. Significant elevations in extracellular dopamine in the medial PFC of rats have been observed in response to sensory cues associated

with food and upon the initiation of food consumption (Ahn & Phillips, 1999; Bassareo & Di Chiara, 1997). Additionally, acute administration of various commonly abused drugs, such as cocaine, amphetamine, alcohol, and THC, has been shown to stimulate extracellular dopamine in the mPFC (Jianping, Paredes, Lowinson, & Gardner, 1990; Moghaddam, Roth, & Bunney, 1990; Sorg & Kalivas, 1993a). Because dopamine signaling facilitates the plasticity underlying reinforcement learning, it is believed that mPFC dopamine mediates some of the reinforcing properties of food and abused drugs. This is supported by studies using conditioned place preference, a behavioral assay of a substance's motivational and reinforcing properties. For example, microinjections of a D1-dopamine receptor antagonists into the medial PFC block cocaine-induced conditioned place preference (Sanchez, Bailie, Wu, Li, & Sorg, 2003). However, a complementary role of mPFC norepinephrine in reward-seeking and consummatory behaviors has not yet been confirmed.

Numerous studies have implicated cortical norepinephrine in mediating attention and arousal, cognitive functions that are involved in appetitive and seeking behaviors. Early in vivo electrophysiological recordings from locus coeruleus neurons in awake monkeys demonstrated an ability of sensory cues, such as the presentation of a juice drink or a food odor, to stimulate neuronal firing (though the projection targets of these neurons were not determined) (Foote, Aston-Jones, & Bloom, 1980). Additionally, some potential mechanisms to facilitate functional crosstalk between noradrenergic and dopaminergic

signaling within the mPFC have been identified. For example, co-localization of D2-dopamine and β1-adrenergic receptors has been observed on prefrontal cortical neurons (Xing, Li, & Gao, 2016). Therefore, it is reasonable to hypothesize that mPFC norepinephrine may play a role in responding to appetitive conditioned stimuli, and some supportive evidence exists. Upon presentation of a conditioned stimulus and a food reward, significant stimulation of extracellular norepinephrine (and dopamine) is observed in the mPFC. Presentation of the conditioned stimulus alone was also sufficient to stimulate extracellular norepinephrine, though this response was much less robust than when food was simultaneously presented (Mingote et al, 2004). In another study, selective lesions of mPFC noradrenergic afferents abolished morphine conditioned place preference in rodents. However, this behavioral effect may have been due to indirect effects on accumbal dopamine signaling, as the same lesions also abolished the morphine-induced dopamine response in the nucleus accumbens (Ventura, Alcaro, & Puglisi-Allegra, 2005). Together, this work indicates a potential role of cortical norepinephrine signaling in appetitive behaviors, which may serve to complement dopamine's role in reward prediction.

mPFC function and alcohol

Some of the cognitive-behavioral functions of the mPFC in which catecholamines have been implicated are affected by acute and chronic alcohol

experience. The effects of acute systemic ethanol on cognitive-behavioral tasks appear to be dose-dependent. Higher doses (1 g/kg and higher) produce impairments in working memory and response inhibition in rodents, while low doses facilitate or have no effect on these measures (Popke, Allen, & Paule, 2000; Rossetti et al., 2002). In humans, while acute ethanol significantly impairs response inhibition, assessing it's effects on working memory has yielded mixed results (Abernathy et al, 2010; Gan et al., 2014; Hendershot et al., 2015). Due to the correlational nature of these acute studies, however, caution must be taken in interpreting these observations as resulting from ethanol's direct actions on mPFC function. A limited amount of work has been done to quantifying the actions of acute ethanol on mPFC neurochemistry and physiology.

In contrast, numerous studies have compared mPFC functioning in detoxified alcoholics and healthy controls. In general, alcoholics typically display hypofunction of the mPFC, which likely contributes to the loss of behavioral control and deficits in executive functions reported in these subjects (Goldstein & Volkow, 2011; Noël et al., 2005; Wang et al., 2016). Relative to controls, human alcoholics show impairments in manipulating working memory, flexibility in planning and problem solving, attentional shifting, ignoring distractions and irrelevant stimuli, and in response inhibition (Noël et al., 2001; Noël et al., 2005; Wang et al., 2016). Additionally, alcoholics show greater activation of the dlPFC and mPFC induced by alcohol-related cues, which is correlated with self-reported craving (Goldstein & Volkow, 2011; Grüsser et al., 2004).

While these studies demonstrate impaired PFC function in human alcoholics, it is often unclear if such deficits are pre-existing or induced by chronic alcohol use. Furthermore, if such deficits are due to ethanol-induced effects on PFC function, it is necessary to determine the mechanisms and time course by which these developments occur. Animal studies have found that synaptic alterations occur in the mPFC with chronic ethanol exposure, particularly among glutamatergic, dopaminergic, and GABAergic signaling, which may contribute to the impairments in mPFC function observed in human and animal models of alcohol dependency (Abernathy et al., 2010; Kroener et al., 2012). For example, following chronic intermittent exposure to ethanol vapor, mice show altered LTP in the mPFC, which was hypothesized to be a result of increased synaptic expression of NR1 and NR2B NMDA receptor subunits (Kroener et al., 2012). Furthermore, these mice displayed deficits in behavioral flexibility, consistent with impaired mPFC activity. However, it is unclear if such synaptic and behavioral changes are apparent following a limited period of voluntary ethanol self-administration, or if these changes are the result of repeated cycles of intoxication and withdrawal.

Additionally, a few studies have demonstrated a functional role of the mPFC in alcohol self-administration. For example, a reduction in responding for ethanol has been reported following microinjections of muscimol, quinpirole or raclopride into the mPFC (Hodge, Chappelle, & Samson, 1996; Samson & Chappell, 2001). Similarly, inactivation/damage to the mPFC impaired extinction

of ethanol conditioned place preference in mice (Groblewski & Cunningham, 2012). Another recent study identified a neuronal ensemble within the infralimbic cortex that appears to regulate cue-induced alcohol seeking behaviors, such that ablation of this ensemble produces persistent alcohol seeking in rats that is not observed following general inactivation of the infralimbic cortex (Pfarr et al., 2015; Willcocks & McNally, 2013). However, the neurochemical correlates of alcohol seeking and consummatory behaviors remain unclear.

METHODS OVERVIEW

Modeling aspects of alcohol use disorders in animals

While animal models do not accurately represent clinical substance use disorders in their entirety, they can be used to examine specific elements of the condition. With regards to the study of alcohol use disorders, animal models are useful for examining the pharmacological, behavioral, neurological, and physiological effects of acute and chronic alcohol experience (Lynch, Nicholson, Dance, Morgan, & Foley, 2010; Tabakoff & Hoffman, 2000). In this dissertation, two different paradigms were employed to examine the effects of ethanol on neurochemistry and the role of catecholamines in ethanol seeking and consummatory behaviors in rats.

1. Passive ethanol administration paradigm

This paradigm is primarily used as a pharmacological assay, as it minimizes the influences of behavior, motivation, and expectancy on neurochemistry. Ethanol may be administered intravenously, intraperitoneally, or intragastrically. Intravenous ethanol administration was employed in the subsequent studies to quantify the acute effects of ethanol on extracellular catecholamines in multiple brain regions.

2. Ethanol self-administration paradigm

   Self-administration paradigms, particularly operant conditioning models, have greater face validity for human alcohol use than passive drug administration paradigms. To facilitate consumption of intoxicating doses of ethanol, outbred strains of rats, including the Long Evans strain used in the subsequent experiments, typically require the use of a sweetened ethanol solution.

Microdialysis

Microdialysis is an analyte collection technique that, when applied to neuroscience, enables the sampling and quantification of neurotransmitters, peptides, hormones, and other molecules from the extracellular space (Chefer, Thompson, Zapata, & Shippenberg, 2009). The central component is the microdialysis probe, which can vary in its construction to optimize recovery of the

analyte of interest. The probes used in the discussed studies were manually constructed per the procedures described by Pettit and Justice (1991). In general, the microdialysis probes consist two pieces of fused silica, an “inlet” and an “outlet” through which analyte-free artificial cerebrospinal fluid (ACSF) is continuously perfused at a low flow rate. At one end, both strands of fused silica are enclosed in a porous cellulose membrane that is semi-permeable on the basis of molecular weight. Once the probe is implanted into the tissue and perfusion begins, analytes diffuse from the extracellular space across the membrane along their concentration gradient (Chefer et al., 2009). Analytes are then picked up by the perfusate and carried through the outlet silica into collection vials. Sample collection times vary depending on the analyte of interest and the sensitivity of the analytical equipment, usually between 1-30 minutes.

In the preceding studies, we have applied in vivo conventional microdialysis to measure the relative extracellular concentrations of ethanol, dopamine, and norepinephrine in awake, freely behaving animals. With conventional microdialysis, the concentration of analyte recovered in the sample vials represents a fraction of the total extracellular concentration. For more details regarding the basic principles of microdialysis, see Chapter 2.

SPECIFIC AIMS AND HYPOTHESES

Overall Rationale

Catecholamine signaling within the dorsal striatum and medial prefrontal cortex mPFC) is critical to the planning and execution of motivated behaviors, including alcohol seeking and consummatory behaviors. Acute and chronic alcohol use have been shown to alter the function and neuronal activity within the dorsal striatum and the mPFC. The direct pharmacological effects of alcohol on catecholaminergic signaling within these regions has not been thoroughly characterized so it is unclear when and by which mechanism ethanol may be inducing alterations. Our objective with the subsequent studies is to quantify neurochemical activity in animal models of initial alcohol exposure in an attempt to understand how ethanol's early pharmacological actions influence or underlie the neuroadaptations observed in animal models of habitual and compulsive alcohol use.

Within the dorsal striatum, dopamine signaling is a critical mediator in the neuroplasticity underlying goal-directed and habitual behaviors, but it is unclear when ethanol-induced plasticity occurs. Furthermore, evidence suggests that differential mechanisms may regulate dopamine signaling within the medial and lateral subregions of the dorsal striatum, which are known to be functionally distinct. Prior to characterizing ethanol-induced alterations in the functions of the

dorsomedial and dorsolateral striata, it is critical to quantify ethanol's acute pharmacological effects on extracellular dopamine within each of these subregions. This may also contribute to our understanding of ethanol's mechanism of action.

The dorsal striatal subregions are a downstream target of mPFC glutamatergic afferents involved in the planning and coordination of motivated behaviors. The activity of glutamatergic neurons within the mPFC has been shown to be modulated by both norepinephrine and dopamine (Goldwater et al., 2009; Sekio & Seki, 2014). Therefore, any observed functional and neuronal alterations in the dorsal striatum may be preceded by altered catecholamine activity within the mPFC. Additionally, it is necessary to understand in which aspects of alcohol seeking and consummatory behaviors that catecholaminergic activity is specifically activated.

Specific aims

Specific aim 1: Quantify and compare the acute pharmacological effects of ethanol on dopamine activity in the dorsomedial and dorsolateral striata

While previous studies have measured dorsal striatal dopamine following an acute administration of ethanol, none have specifically explored subregional differences. Given the functional and anatomical distinctions of the dorsomedial

and dorsolateral striatum, we predict that ethanol would have differential effects on extracellular dopamine in these subregions.

Specific aim 2: Validate the emergence of a hypodopaminergia in the mPFC with limited, voluntary ethanol self-administration experience.

Following about one week of sweetened ethanol self-administration, rats showed reduced basal dopamine concentrations in the mPFC compared to control rats that received sucrose or no reinforcer. Although this observation is consistent with previous work associating a hypodopaminergic state with high alcohol consumption and animal models alcohol of dependence (S. R. George et al., 1995; Karkhanis, Rose, Huggins, Konstantopoulos, & Jones, 2015; Siciliano et al., 2016), such a state has not been observed following only one week of ethanol experience in non-dependent animal models. Therefore, replicating our findings is necessary.

Specific aim 3: Quantify extracellular norepinephrine activity in the mPFC following acute, intravenous ethanol and during an operant self-administration session

While ethanol's interactions with the mesocortical dopamine system have been thoroughly explored, the cortical noradrenergic system has received considerable less attention. Given norepinephrine's role in regulating arousal, and that initial exposure to ethanol can be acutely arousing, we expect to

observe an increase in extracellular norepinephrine concentrations following acute, intravenous ethanol administration. Additionally, within the mPFC, extracellular concentrations of dopamine and norepinephrine are similarly regulated, and they appear to work in concert to modulate executive functions and motivated behaviors. Therefore, we will quantify the temporal activity of extracellular mPFC using the same operant training paradigm applied in Aim 2.

Chapter 2:

Temporal profiles dissociate regional extracellular ethanol vs. dopamine concentrations

The work presented in this chapter was published as a review in 2015 in ACS Chemical Neuroscience by Vena and Gonzales (see http://pubs.acs.org/doi/10.1021/cn500278b). It is reprinted here with permission from American Chemical Society (http://pubs.acs.org/page/policy/authorchoice_termsofuse.html). This manuscript and the analyses contained herein were conducted by Ashley Vena.

ABSTRACT

In vivo monitoring of dopamine via microdialysis has demonstrated that acute, systemic ethanol increases extracellular dopamine in regions innervated by dopaminergic neurons originating in the ventral tegmental area and substantia nigra. Simultaneous measurement of dialysate dopamine and ethanol allows comparison of the time courses of their extracellular concentrations. Early studies demonstrated dissociations between the time courses of brain ethanol concentrations and dopaminergic responses in the nucleus accumbens (NAc) elicited by acute ethanol administration. While both brain ethanol and extracellular dopamine levels peak during the first 5 minutes following systemic ethanol administration, the dopamine response returns to baseline while brain ethanol concentrations remain elevated. *Post hoc* analyses examined ratios of the dopamine response (represented as a percent above baseline) to tissue

concentrations of ethanol at different time points within the first 25-30 minutes in the prefrontal cortex, NAc core and shell, and dorsomedial striatum following a single intravenous infusion of ethanol (1 g/kg). The temporal patterns of these "response ratios" differed across brain regions, possibly due to regional differences in the mechanisms underlying the decline of the dopamine signal associated with acute intravenous ethanol administration and/or to the differential effects of acute ethanol on the properties of subpopulations of midbrain dopamine neurons. This review draws on neurochemical, physiological, and molecular studies to summarize the effects of acute ethanol administration on dopamine activity in the prefrontal cortex and striatal regions, to explore the potential reasons for the regional differences observed in the decline of ethanol-induced dopamine signals, and to suggest directions for future research.

INTRODUCTION

Alcoholism represents the end stage in the transition from voluntary to uncontrolled alcohol consumption. These behavioral transitions are the result of ethanol-induced alterations in the fundamental molecular and cellular processes that regulate cognition, motivation, and reward seeking behaviors. Therefore, characterizing the acute neurochemical effects of ethanol is critical to understanding the development and progression of alcohol use disorders.

Ethanol is believed to exert its reinforcing effects on behavior, at least in part, via activation of the mesolimbic dopamine circuit. This circuit consists of dopamine neurons originating in the ventral tegmental area (VTA) and terminating in the nucleus accumbens (NAc), and is implicated in motivated and goal-directed behaviors (Gonzales, Job, & Doyon, 2004). Ethanol has been shown to acutely enhance the firing rate of VTA dopamine neurons *in vitro* and increase extracellular dopamine in the NAc in awake, freely moving animals (Brodie, Pesold, & Appel, 1999; Brodie, Shefner, & Dunwiddie, 1990) (for reviews see (Gonzales et al., 2004; Morikawa & Morrisett, 2010)). Additional pharmacological, lesion, and genetic studies have further implicated the mesolimbic dopamine circuit as a target for ethanol (Gonzales et al., 2004; Koob & Volkow, 2010; Morikawa & Morrisett, 2010; Westerink, Enrico, Feimann, & De Vries, 1998).

Additionally, ethanol has been shown to effect mesocortical and nigrostriatal dopamine activity. Mesocortical dopamine neurons originate in the VTA and terminate in the prefrontal cortex (PFC), and contribute to the regulation of cognition and executive control of goal-directed behaviors (Westerink et al., 1998). Nigrostriatal dopamine neurons originate in the substantia nigra and innervate the dorsal striatum. These neurons coordinate motor responses relevant to goal-directed and habitual behaviors (Koob & Volkow, 2010; Santiago & Westerink, 1992; Yin & Knowlton, 2006). Neurochemical studies demonstrate that acute ethanol administration results in increased extracellular dopamine levels in the prefrontal cortex (Z.-M. Ding et al., 2011; Schier, Dilly, & Gonzales,

2013). In contrast, the nigrostriatal dopamine circuit may be less sensitive to acute ethanol administration (Di Chiara & Imperato, 1988; Melendez, Rodd-Henricks, McBride, & Murphy, 2003), but may be gradually recruited with chronic ethanol self-administration (Budygin et al., 2003, 2007; Corbit, Nie, & Janak, 2014; Koob & Volkow, 2010).

This review summarizes recent *in vivo* microdialysis studies exploring the effects of acute, passive ethanol administration on dopamine activity in the medial PFC and striatal subregions. Additionally, we conducted post hoc analyses on these published and unpublished data to explore the decline of the ethanol-induced dopamine signal during the descending limb of the ethanol concentration time course in the medial PFC, NAc core and shell, and dorsomedial striatum (DMS). The results of our analyses revealed unexpected differences across these regions. In this review, we discuss the rationale and methodology for the post hoc analyses, propose explanations for the observed regional differences, and suggest directions for further research.

DISSOCIATION OF THE TEMPORAL PROFILES OF DIALYSATE ETHANOL AND DOPAMINE

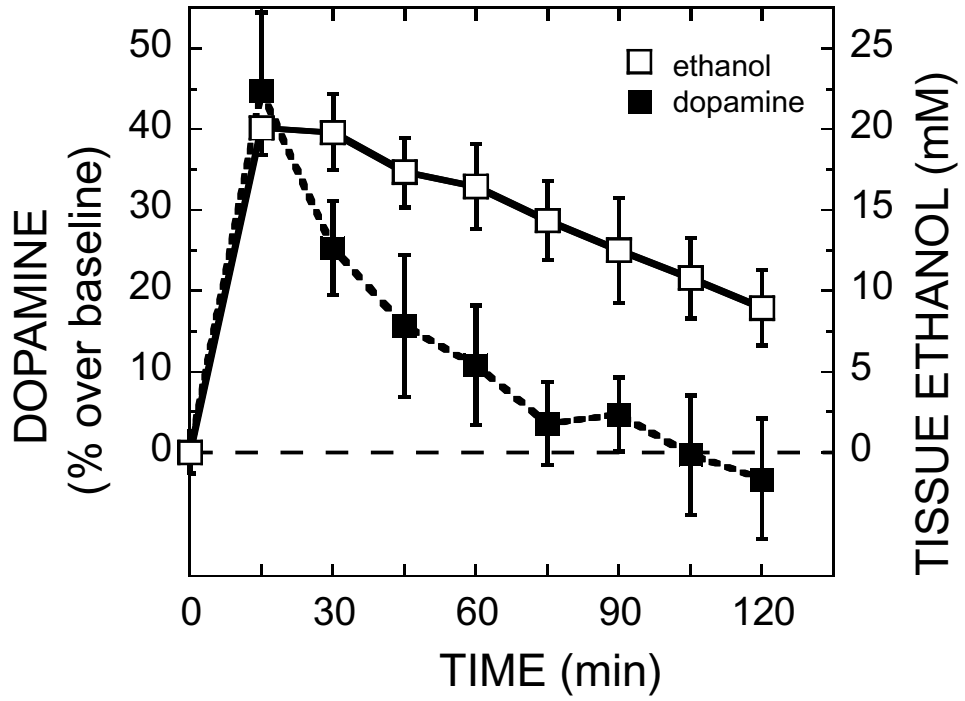
In vivo microdialysis is frequently employed to monitor and quantify extracellular neurochemical changes in select brain regions induced by pharmacological, behavioral, or environmental manipulations in freely moving animals (Gonzales, Tang, & Robinson, 2002) Over the past few decades,

changes in extracellular dopamine activity in response to acute ethanol have been extensively investigated using *in vivo* molecular monitoring techniques, including microdialysis. While the temporal resolution of microdialysis is limited, this technique can detect relatively fast changes in extracellular concentrations of various analytes with sampling times as low as 1 minute (Newton & Justlce, 1994).

Our lab and others have extended its application to monitor the quantity and time course of brain concentrations of ethanol following systemic administration (Ferraro, Weyers, Carrozza, & Vogel, 1990; Howard, Schier, Wetzel, Duvauchelle, & Gonzales, 2008; Schier et al., 2013; Valenta et al., 2013; Yoshimoto & Komura, 1993). Concurrent analyses of both analytes from the same microdialysis sample enables characterization of dopaminergic activity relative to ascending and descending tissue concentrations of ethanol. Using this approach, it was discovered that the time course of the dopamine response in the NAc to acute ethanol did not overlap with the temporal profile of brain ethanol concentrations (Fig. 1) (Yim et al., 2000). Yim et al. reported that following an intraperitoneal (i.p.) injection of 1 or 2 g/kg ethanol in naïve rats, extracellular dopamine reaches peak concentrations to 140% of baseline levels within the first 15-minute sample while ethanol also attains peak brain concentrations 15-30 minutes following the injection, depending on the dose administered. The accumbal dopamine response returns to baseline 60-90 minutes post injection, while ethanol remains elevated in the dialysate. Dialysate ethanol concentrations

did not return to baseline during the 2-hour sampling period post-injection (Yim et al., 2000).

Figure 1: Dialysate concentrations of dopamine in the nucleus accumbens and tissue concentrations of ethanol following acute ethanol administration (1 g/kg, i.p.).



There is a dissociation in the time courses of dopamine and ethanol concentrations in which dopamine returns to baseline levels while ethanol remains elevated in the tissue. The ethanol injection occurred at 0-minute time point. Symbols represent mean ± s.e.m (n=5). Data from Yim et al. (2000).

Interpreting the dissociation of the temporal profiles of dialysate ethanol and dopamine: relevance to acute tolerance

Yim et al. hypothesized that the observed dissociation in the time courses of the dopamine response and dialysate ethanol concentrations following acute ethanol administration may be due to the development of acute tolerance (Yim et al., 2000). The dissociation between ethanol and dopamine occurs during the descending phase of the brain ethanol concentration curve, and this temporal pattern aligns with that observed in behavioral studies of acute tolerance in humans and rodents (Le & Mayer, 1995; LeBlanc, Kalant, & Gibbins, 1975). Following a single dose of ethanol in humans, behavioral stimulation is reported during the ascending limb of the blood ethanol curve, while sedation and reduced impairments in the activation of motor responses are reported during the descending limb of the blood ethanol curve (Fillmore, Marczinski, & Bowman, 2005; Martin, Earleywine, Musty, Perrine, & Swift, 1993). Acute tolerance to the stimulating and motor impairing effects of ethanol represents a physiological adaption occurring during a single ethanol exposure (Le & Mayer, 1995; LeBlanc et al., 1975), and may be relevant in predicting individual vulnerability to alcohol use disorders (Fillmore et al., 2005; Martin et al., 1993; Tampier, Quintanilla, & Mardones, 2000). For example, selectively bred alcohol-preferring rats develop acute tolerance to a single dose of ethanol more rapidly than non-preferring rats

(Tampier & Mardones, 1999; Waller, McBride, Lumeng, & Li, 1983). Consistent with this observation, rats displaying high acute tolerance tend to consume larger quantities of ethanol (Tampier et al., 2000). Together these findings suggest a relationship between the propensity to consume large quantities of ethanol (possibly due to a genetic vulnerability) and the tendency to exhibit rapid acute behavioral tolerance.

In alcohol non-preferring rats, acute tolerance to the motor impairing effects of ethanol develops within 60-90 minutes following an i.p. injection of 2 or 2.3 g/kg ethanol (Tampier & Mardones, 1999; Waller et al., 1983). This time course overlaps with that of the dissociation between ethanol and dopamine following an i.p. injection of a 1 g/kg dose of ethanol (Yim et al., 2000). While dopamine in the NAc likely is not responsible for the specific motor behaviors assessed in the studies by Tampier et al. (1999; 2000) and Waller et al. (1983), dopaminergic mechanisms are hypothesized to contribute to the acute stimulating effects of low to moderate doses of ethanol during the ascending limb of the blood ethanol concentration curve (Imperato & Di Chiara, 1986; Wise & Bozarth, 1987). Early work showed that following i.p. administration of 0.25 and 0.5 g/kg ethanol, peak behavioral stimulation (defined as rearing, ambulation, and grooming) correlated with peak extracellular dopamine activity in the NAc at 20 minutes post-injection, and behavioral activity declined as dopamine levels returned to baseline (Imperato & Di Chiara, 1986). Additionally, dopamine antagonists have been shown to dose-dependently reduce the locomotor-

stimulating effects of ethanol in FAST mice, a strain of mice that is highly sensitive to the stimulating effects of acute ethanol (Shen, Crabbe, & Phillips, 1995). However, while dopaminergic mechanisms may contribute to the expression of acute tolerance, the exact cellular and molecular mechanisms underlying this phenomenon are unknown, and therefore one cannot rule out the possibility of additional contributory mechanisms outside of the mesolimbic dopamine system (Ludvig, George, Tang, Gonzales, & Bungay, 2001).

Interpreting the dissociation of the temporal profiles of dialysate ethanol and dopamine: relevance to ethanol's mechanism of action

A temporal dissociation between extracellular dopamine and drug concentrations is not observed with psychostimulants but has been observed with morphine. These effects may be related to differences in the mechanisms of actions of ethanol, psychostimulants, and morphine. Following acute drug administration, psychostimulants demonstrate a direct relationship between brain concentrations of drug and the dopamine response in the striatum. Using *in vivo* microdialysis, Kuczenski et al. (1997) demonstrated that extracellular concentrations of striatal dopamine and amphetamine showed nearly identical temporal profiles following a single subcutaneous dose of amphetamine (Fig. 2a) (Kuczenski et al., 1997). A similar concentration-response relationship has been observed with cocaine. Following an i.p. injection of 30 mg/kg, cocaine attains a

maximum concentration of 10 micromolar within 20-30 minutes post injection (Fig. 2b) (Nicolaysen, Pan, & Justice, 1988). Extracellular dopamine concentrations in the striatum also peaked at 30 minutes post cocaine administration. As extracellular concentrations of cocaine and dopamine declined, there was a linear relationship between dialysate dopamine and drug concentrations (Nicolaysen et al., 1988). The effect of cocaine on extracellular dopamine has also been shown to occur within seconds of an intravenous infusion using fast scan cyclic voltammetry (Cheer et al., 2007; Mateo, Budygin, Morgan, Roberts, & Jones, 2004), but this method does not allow concurrent analysis of extracellular cocaine concentrations; therefore, the relationship between the drug response and the drug under these conditions is not completely clear.

Interestingly, in contrast to psychostimulants, a dissociation in dialysate concentrations of morphine and extracellular dopamine in the striatum occurs following acute administration of morphine. However, the time course of this dissociation contrasts with that of ethanol in that the dissociation between extracellular concentrations of morphine and dopamine appears to occur primarily during ramping up of the dopamine response, rather than during the decline of the dopamine response. Gottas et al. recently demonstrated that following intravenous (i.v.) morphine administration, drug concentrations in the brain reached peak levels within 5-7 minutes (Fig. 2c) (Gottås, Boix, Øiestad, Vindenes, & Mørland, 2014). In contrast, extracellular dopamine in the striatum

gradually increased, reaching peak levels approximately 46 minutes following the i.v. morphine infusion. Thereafter, extracellular morphine and dopamine levels slowly declined towards baseline, but neither reached baseline during the 2 hours following the infusion. During the decline of the dopamine signal, the dissociation with extracellular concentrations of morphine was less apparent (Gottås et al., 2014).

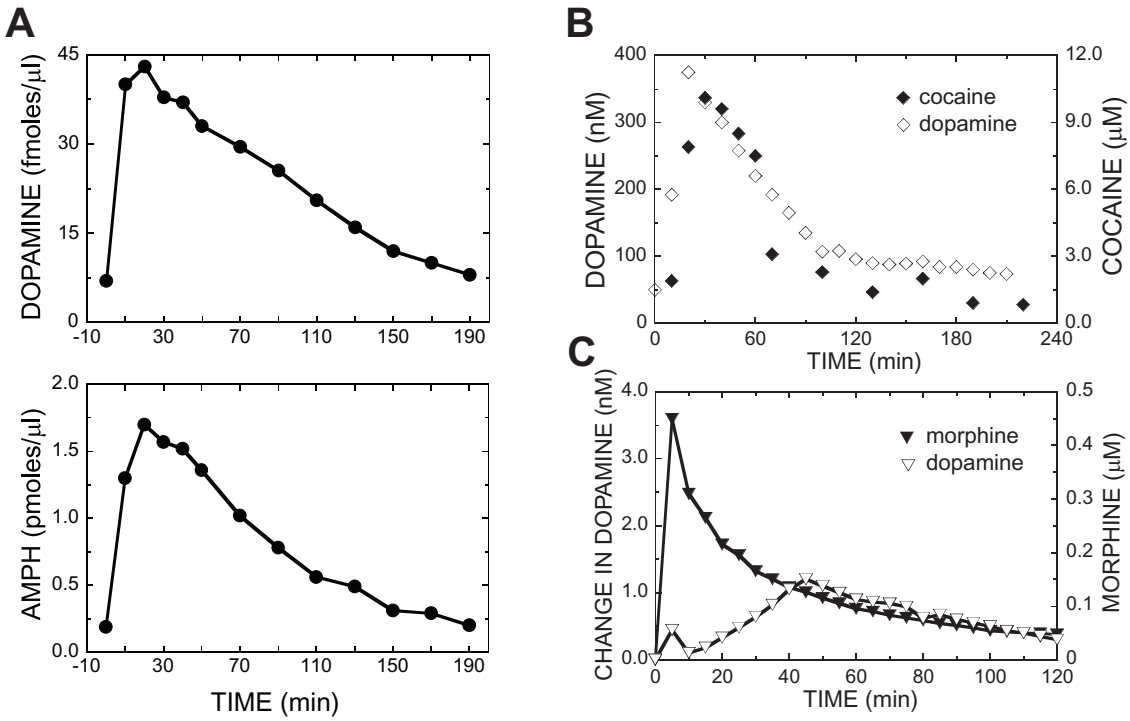
The mechanisms by which psychostimulants and morphine enhance extracellular dopamine are well understood. Cocaine and amphetamine exert their primary effects on dopamine activity at the terminals of dopamine neurons. Cocaine inhibits the dopamine transporter, blocking a major mechanism of dopamine clearance from the synapse and thus, resulting in increased levels of extracellular dopamine (Church, Justice, & Byrd, 1987; Nicolaysen et al., 1988). Amphetamine also alters the function of the dopamine transporter in addition to interfering with the storage of dopamine into synaptic vesicles (Calipari & Ferris, 2013). In contrast, the molecular and cellular mechanisms by which ethanol enhances dopaminergic activity are not clearly understood. The lack of a direct relationship between extracellular ethanol and dopamine is consistent with experimental evidence that ethanol does not directly impair dopamine reuptake (Budygin, Phillips, Wightman, & Jones, 2001; Yim & Gonzales, 2000). Using no net flux *in vivo* microdialysis, it was demonstrated that a 1-g/kg (i.p.) dose of ethanol increases the equilibrium point where no net flux is observed for

dopamine in the NAc, but it does not alter the slope of the no net flux plot (Yim & Gonzales, 2000).

Alternative possibilities include an indirect effect of ethanol on the stimulation of dopamine release or a rapid desensitization of the mechanism(s) by which ethanol acts to facilitate increased dopaminergic activity. A mechanism by which morphine increases mesocorticolimbic dopamine activity is through binding to mu opioid receptors (MORs) on specific GABAergic terminals that synapse onto VTA dopamine neurons. Activation of these MORs hyperpolarizes the GABA neuron, removing the tonic inhibition of VTA dopamine neurons (Di Chiara & North, 1992; Johnson & North, 1992; Volman et al., 2013). The possibility of a disinhibitory mechanism of ethanol action on VTA dopamine neurons has been suggested based on evidence demonstrating a reduction in the activity of VTA GABAergic neurons following ethanol administration (Gallegos, Lee, Criado, Henriksen, & Steffensen, 1999; Stobbs et al., 2004; Xiao, Zhang, Krnjević, & Ye, 2007). However, it is not entirely clear if this effect underlies the stimulation of mesocorticolimbic dopamine activity observed *in vivo* following acute ethanol administration (for review see (Morikawa & Morrisett, 2010)). Furthermore, other groups have reported conflicting results regarding the effect of ethanol on GABAergic transmission in the VTA. For example, ethanol has been shown to potentiate GABA release onto VTA dopamine neurons *in vitro* (Theile, Morikawa, Gonzales, & Morrisett, 2008, 2009). Additionally, a recent microdialysis study showed no significant effect of systemic ethanol

administration on GABA concentrations in the VTA of alcohol-preferring and alcohol non-preferring rat lines (Kemppainen, Raivio, Nurmi, & Kiianmaa, 2010).

Figure 2: Temporal profiles of extracellular concentrations of amphetamine, cocaine, or morphine and dopamine following acute systemic administration.



A) Extracellular concentrations of dopamine (top left panel) and amphetamine (bottom left panel) in the dorsal striatum demonstrate nearly identical temporal profiles following acute administration of amphetamine (8 mg/kg, s.c.). Symbols represent mean. Reproduced with permission from Kuczenski et al. (1997). B) Extracellular concentrations of cocaine (filled diamonds) and dopamine (open diamonds) in the striatum demonstrate similar temporal profiles following cocaine administration (30 mg/kg, i.p.). Symbols represent mean. Reproduced with permission from Nicolaysen et al. (1988). C) A temporal dissociation in dialysate concentrations of morphine (filled triangles) and extracellular dopamine (open triangles) in the striatum occurs within the first 40 minutes following acute administration of morphine (1 mg, i.v.). After 40 minutes, a temporal dissociation is no longer apparent between extracellular levels of morphine and dopamine. Symbols represent mean. Reproduced with permission from Gottas et al. (2014).

"RESPONSE RATIOS"

The original study by Yim et al. directly compared the time courses of the dopamine response and dialysate ethanol concentrations, focusing on an extended time period encompassing the 15-120 minutes following the intraperitoneal (i.p.) injection (Yim et al., 2000). This allowed comparison with ethanol-induced behaviors that had similar time courses, as discussed above. More recent work has now allowed higher resolution sampling during the microdialysis experiment so that times within the first 30 minutes of ethanol administration can be analyzed.

A potential confound in the study by Yim et al. is that ethanol administration via i.p. injections may be aversive to naïve rats and as a result, such studies may include effects of stress on dopamine activity (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Ciccocioppo et al., 1999; Cloutier & Newberry, 2008; Kalivas & Duffy, 1995; Sorg & Kalivas, 1993b). Intravenous ethanol administration minimizes stress in naïve animals because no animal handling is required. Using this route of administration, Howard et al. found a similar dissociation in the decline of the dopamine response relative to descending concentrations of ethanol (Howard et al., 2008).

To explore the dissociation in the temporal profiles of extracellular dopamine and brain ethanol concentrations across brain regions, we performed post hoc analyses on our existing body of data. Similar to Yim et al. we computed ratios (referred to as "response ratios") of the dopamine response

(represented as a percent over baseline) to tissue concentrations of ethanol (Yim et al., 2000). We hypothesized that within the first 25-30 minutes following acute ethanol administration, the “response ratios” within each brain region would decline in a similar manner. Contrary to our expectations, we observed regional differences in the temporal profiles of the “response ratios”, suggesting distinct mechanisms may underlie the decline of the dopamine signal during the descending limb of the ethanol concentration curve. Here we describe the methods by which we determined the “response ratios” for each brain region, our results, and a limited interpretation of our results.

We analyzed data collected from *in vivo* microdialysis experiments in the nucleus accumbens core (NAc core) and shell (NAc shell) regions, medial prefrontal cortex (mPFC), and dorsomedial striatum (DMS) following acute i.v. ethanol administration (1 g/kg). In the subsequent sections, we first review the methodological details of our microdialysis experiments and discuss the adjustments made to our calculations to correct for procedural differences across experiments. Due to the lower concentration of endogenous dopamine in the mPFC, methodological modifications, such as an increase to probe lengths and a decrease in the perfusate flow rate, were made to enhance dopamine recovery in this region.

The probes used in our studies are constructed in our laboratory according the procedures described by Pettit and Justice (Doyon et al., 2003a; Howard et al., 2008; Pettit & Justice, 1991a; Schier et al., 2013; Valenta et al.,

2013). The probe active area is 1.5 millimeters for striatal regions and 2.75-3.25 millimeters for the mPFC (Howard et al., 2008; Schier et al., 2013; Valenta et al., 2013). Probes are continuously perfused with artificial cerebrospinal fluid (ACSF) at a flow rate of 2 microliters/minute for striatal samples and 1 microliter/minute for prefrontal cortical samples (Howard et al., 2008; Schier et al., 2013; Valenta et al., 2013). In every experiment, 2-4 samples are collected prior to any infusions to determine basal dopamine levels for each animal. Relative standard deviations are calculated to assess the stability of basal dopamine activity for each animal. Only those animals demonstrating relative standard deviation values <0.25 were included in the microdialysis experiments. In striatal experiments, samples are collected in 5-minute intervals, but the collection time is increased to 10 minutes for mPFC samples to account for the decreased flow rate. To control for any effects of an i.v. infusion on extracellular dopamine activity, a saline infusion is given either to the same animal prior to the ethanol infusion (for within-subjects study designs) or to a separate group of animals (for between-subjects study designs) and dialysate samples are subsequently collected. The control saline infusions had no significant effects on extracellular dopamine in all of the experiments included in our analyses (Howard et al., 2008; Schier et al., 2013; Valenta et al., 2013). At the conclusion of experiments, the ACSF is replaced with calcium-free ACSF and perfused through the probe for 1-2 hours and a final 2 samples are collected. These samples are necessary to

confirm calcium-dependent exocytotic dopamine release from neurons surrounding the probe membrane.

In vivo extraction fraction for ethanol

Dialysate ethanol concentrations are quantified via gas chromatography, but these concentrations are only a fraction of the tissue concentration of ethanol. To determine the *in vivo* recovery of ethanol for our probes in Long Evans rats, Howard et al. inhibited ethanol metabolism via intravenous administration of the alcohol dehydrogenase inhibitor 4-methylpyrazole (2 mg/kg) to produce a “pseudo-steady state”, and then systemically administered ethanol. A ratio of dialysate ethanol concentrations to blood ethanol concentrations was calculated and the *in vivo* extraction fraction for ethanol was determined to be 0.14 (Howard et al., 2008). This value was used to determine the tissue concentrations of ethanol for each animal included in our analyses.

Effect of methodological differences on in vivo ethanol recovery

To account for the differences in microdialysis parameters across experiments, we made adjustments to our calculations of ethanol tissue concentrations. A linear relationship approximates the increase in ethanol recovery across a probe as a function of probe length in the range of 1-3 mm

(George, 2000). Therefore, the *in vivo* recovery constant for ethanol was adjusted accordingly for the mPFC data. For example, the extraction fraction for ethanol for a probe with a length of 3 millimeters would be doubled to 0.28. Additionally, the microdialysis experiments sampling from the mPFC used a lower perfusate flow rate than the striatal experiments. An inverse relationship exists between perfusate flow rate and analyte extraction fraction, where the percent of relative *in vivo* recovery declines exponentially as the flow rate is increased (Benveniste, 1989; Chefer, Thompson, Zapata, & Shippenberg, 2009; Jamal et al., 2003; Kendrick, 1989). As a result, the *in vivo* extraction fraction for ethanol was also increased by a factor of 1.56 for animals in the mPFC experiments. Therefore, with both adjustments accounting for the increased probe length and decreased flow rate, the final extraction fraction for ethanol for the mPFC dialysate samples was 0.364-0.437.

Methods

Using a similar method to that described by Yim et al. (2000), we calculated tissue concentrations of ethanol and “response ratios” for each animal within the first 25-30 minutes following the ethanol infusion (1 g/kg). It should be noted that because the studies were not conducted simultaneously, we are unable to directly compare the “response ratios” across the four brain regions. Given the variability in basal dopamine levels across the NAc core and shell,

mPFC, and DMS, we focused on the percent change in dopamine levels relative to baseline. However, we also conducted the same analyses on the raw dopamine values and obtained similar temporal patterns in the “response ratios” for each brain region (data not shown). The equations used to determine tissue concentrations of ethanol and “response ratios” are listed below:

$$tissue\ [etoh] = \frac{dialysate\ [etoh]}{extraction\ fraction}$$

$$response\ ratio = \frac{dopamine\ response}{tissue\ [etoh]}$$

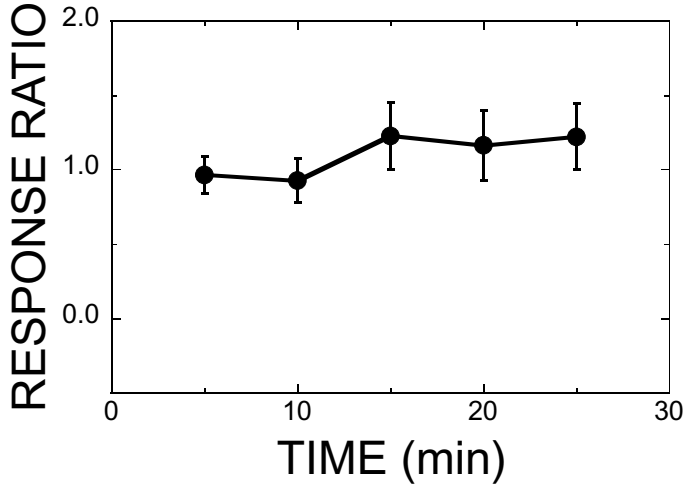
“Response ratios” for NAc core and shell

Following i.v. ethanol administration, the dopamine response in the NAc shell peaked to 40% over baseline within the first 5-minute sample and then declined faster than dialysate ethanol concentrations. For the “response ratio” analyses, 23 animals from 3 studies (Howard et al., 2008; Valenta et al., 2013) (the third study is unpublished) were included. It should be noted that a subset of these animals (n=5) received a hypotonic ethanol solution, though it is unlikely that this had any significant effects on extracellular dopamine in the NAc, as hypotonic and isotonic ethanol solutions produced no differential effects on extracellular dopamine in the mPFC (Schier et al., 2013). There were no

statistically significant changes in the “response ratios” the initial 25 minutes following the ethanol infusion for the NAc shell (Fig. 3; $F(4, 88)=1.82$, n.s.). Of the animals included in the analyses, there were 4 animals whose dopamine response returned to or dropped below baseline within the 25 minutes following the ethanol infusion.

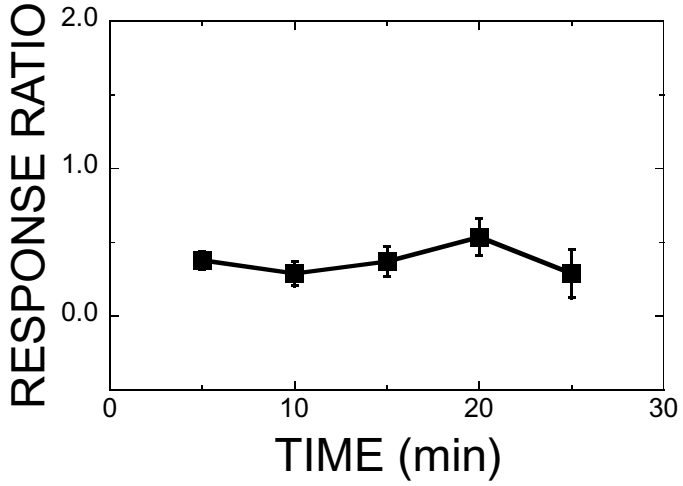
For the NAc core, 6 animals from one study (Howard et al., 2008) were included in the “response ratio” analyses, and these animals also received a hypotonic ethanol solution. Within the first 25 minutes following the ethanol infusion, there were no significant changes in the “response ratios” in the core (Fig. 4; $F(4, 20)=1.05$, n.s.). The ethanol-induced dopamine response returned to or dropped below baseline in 2 of the 6 animals within 25 minutes following the ethanol infusion. Three additional animals had extracellular dopamine levels return to near baseline levels within the last 5-minute sample.

Figure 3: “Response ratios” in the NAc shell region for the first 25 minutes following intravenous ethanol administration (1-g/kg).



The ratios are the dopamine response (represented as a percent over baseline) relative to tissue concentrations of ethanol. Symbols represent mean ± S.E.M (n=23).

Figure 4: “Response ratios” in the NAc core region for the first 25 minutes following intravenous ethanol administration (1-g/kg).



The ratios are the dopamine response (represented as a percent over baseline) relative to tissue concentrations of ethanol. Symbols represent mean ± S.E.M (n=6).

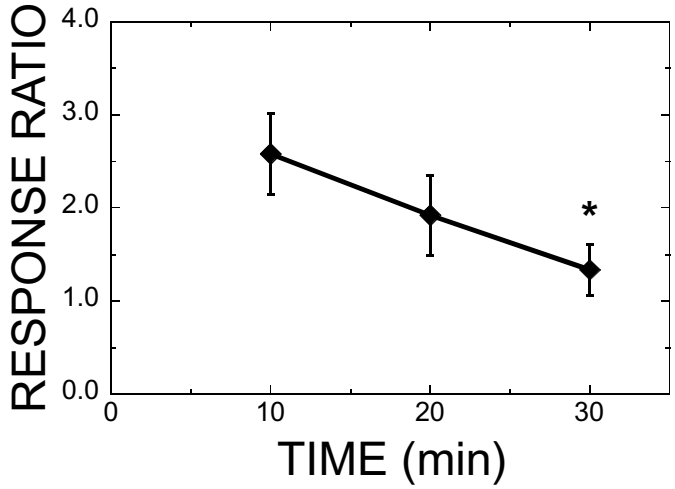
“Response ratios” for mPFC

Nineteen animals from one study (Schier et al., 2013) were included in the “response ratio” analyses for the mPFC. The “response ratios” significantly declined at a relatively linear rate over the first 30 minutes following the ethanol infusion (Fig. 5; $F(2, 36)=5.66$, $p=0.007$). In 3 of the 19 animals included in the analyses, the dopamine response returned or dropped below baseline within the 30 minutes following the ethanol infusion.

“Response ratios” for DMS

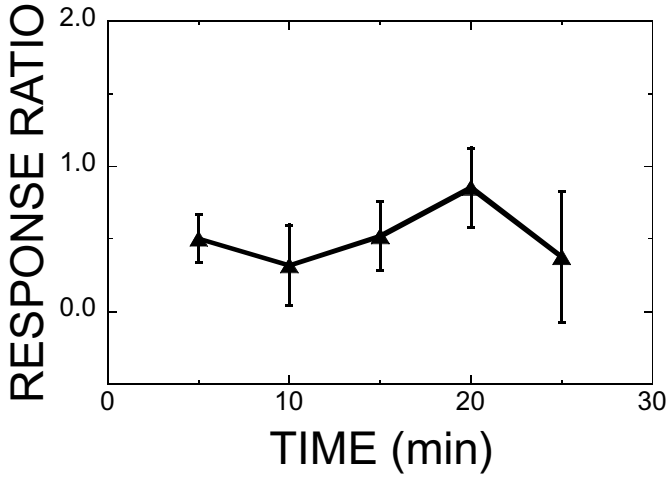
The DMS “response ratio” analyses included 9 animals from one study (unpublished data). There was no main effect of time in the overall ANOVA for the “response ratios” in this region (Fig. 6; $F(4, 32)=0.553$, n.s.). There were 5 animals whose dopamine responses returned to or dropped below baseline within the first 25 minutes following the ethanol infusion.

Figure 5: “Response ratios” in the PFC region for the first 30 minutes following intravenous ethanol administration (1-g/kg).



The ratios are the dopamine response (represented as a percent over baseline) relative to tissue concentrations of ethanol. Symbols represent mean ± S.E.M (n=19). *Post hoc T-tests indicate significance when compared to the 10-minute time point following overall significance in the ANOVA; $p<0.05$.

Figure 6: “Response ratios” in the DMS region for the first 30 minutes following intravenous ethanol administration.



The ratios are the dopamine response (represented as a percent over baseline) relative to tissue concentrations of ethanol. Symbols represent mean ± S.E.M (n=9).

INTERPRETATION

"Response ratios" were calculated for the first 25-30 minutes following acute i.v. ethanol and thus are likely not relevant to acute behavioral tolerance, as behavioral tolerance occurs on the time course of hours, as discussed above. However, within this short time frame, acute tolerance may be developing to the pharmacological mechanisms by which ethanol stimulates mesocorticolimbic dopamine activity. Our analyses of "response ratios" do not directly assess the mechanism by which ethanol stimulates extracellular dopamine concentrations. However, these analyses did reveal interesting regional differences in the decline of the dopamine signal during the descending limb of the blood ethanol concentration curve. Below, we speculate about possible reasons for the faster decline in the "response ratios" in the PFC versus striatal regions.

Projection-specific subpopulations of midbrain dopamine neurons may be differentially affected by ethanol

Recent work has suggested that midbrain dopamine neurons are physiologically, molecularly and functionally distinct, and therefore may be differentially affected by commonly abused drugs. While there is not yet a consensus in the field regarding the specific differences among midbrain dopamine neurons, and species-specific differences are apparent, recent work has demonstrated that specific characteristics of midbrain dopamine neurons

vary depending on neuronal projection targets. Some of this recent work, as well as the general physiological and molecular characteristics of midbrain dopamine neurons have been reviewed previously (Lammel, Lim, & Malenka, 2014; Marinelli & McCutcheon, 2014; Morikawa & Morrisett, 2010; Ungless & Grace, 2012; Volman et al., 2013; Yetnikoff, Lavezzi, Reichard, & Zahm, 2014) and thus will be only briefly summarized here.

Specifically, VTA dopamine neurons projecting to the PFC, NAc core, NAc medial shell, and basolateral amygdala (BLA) do not universally display the characteristics historically used to identify dopamine neurons. For example, recordings from adult mouse brain slices demonstrate that in response to low current levels, these dopamine neurons fire action potentials at frequencies in the range of 10-15 Hz, which are significantly higher than the firing frequencies of those projecting to the NAc lateral shell and nigrostriatal dopamine neurons (3-6 Hz) *in vitro* (Lammel et al., 2008, 2014; Roeper, 2013; Ungless & Grace, 2012). Furthermore, these fast-firing dopamine neurons are able to sustain these higher firing frequencies for several seconds (Lammel et al., 2008). Another key physiological difference is the lack of an I_h current in the fast-firing dopamine neurons *in vitro*, which contrasts the large I_h current observed in dopamine neurons projecting to the NAc lateral shell (Lammel et al., 2014; Ungless & Grace, 2012).

Additionally, molecular differences exist among these distinct subpopulations of midbrain dopamine neurons, including the expression of

somatodendritic D2-like autoreceptors, which has historically been used as a criterion for identifying dopamine neurons. Using transgenic mice that lacked specifically D2-subtype autoreceptors on dopamine neurons, but expressed postsynaptic D2 receptors on non-dopaminergic neurons, Bello et al. recorded the activity of presumed midbrain dopamine neurons in horizontal brain slices (Bello et al., 2011). These neurons did not respond to bath application of quinpirole, while those from control mice demonstrated hyperpolarization. This work provides strong evidence that within the D2-like receptor family, D2-subtype receptors are the primary mediators of auto-inhibition at the level of the cell body in midbrain dopamine neurons (Bello et al., 2011). However, the projection targets of the recorded neurons were not identified, which is critical given the profound heterogeneity observed among midbrain dopamine neurons. Furthermore, the identification criteria for dopamine neurons used by Bello and colleagues may have prevented sampling from mesocortical neurons, which appear to lack somatodendritic autoreceptors altogether (Bannon, Michaud, & Roth, 1981; Bannon & Roth, 1983; Lammel et al., 2008; Ungless & Grace, 2012). Lammel and colleagues reported that in coronal midbrain slices of adult mice, bath application of 100 mM dopamine did not alter the firing frequencies of mesocortical dopamine neurons, while hyperpolarizing all other VTA dopamine neurons (Lammel et al., 2008). It should be stated, however, that species-specific variation may exist with regards to the expression of somatodendritic autoreceptors on mesocortical dopamine neurons. Margolis and colleagues

identified PFC-projecting tyrosine hydroxylase-positive neurons that were hyperpolarized by bath-application of quinpirole in horizontal brain slices from adolescent rats (Margolis, Mitchell, Ishikawa, Hjelmstad, & Fields, 2008).

When considering these molecular and physiological distinctions, it is not surprising that midbrain dopamine neurons also demonstrate significant pharmacological and functional heterogeneity that is also associated with their projection targets. In a series of studies, Westerink and colleagues (Santiago & Westerink, 1992; Westerink et al., 1998; Westerink, Kwint, & deVries, 1996) demonstrated significant differential responsiveness of mesocortical, mesolimbic, and nigrostriatal dopamine neurons to various pharmacological manipulations. For example, infusion of the $GABA_a$ receptor agonist muscimol into the VTA through a microdialysis probe significantly decreased extracellular dopamine in the PFC and NAc, but in contrast, muscimol infused into the SNc significantly elevated extracellular dopamine levels in the dorsal striatum (Westerink et al., 1998; Westerink et al., 1996). Administration of NMDA and the $GABA_b$ receptor agonist baclofen into the VTA via a microdialysis probe also produced differential effects on the percent change in and the temporal pattern of extracellular dopamine in the PFC, NAc, and dorsal striatum (Westerink et al., 1998).

Rewarding and aversive stimuli also have been shown to produce differential effects on extracellular dopamine in cortical and striatal regions. Acute exposure to rewarding or appetitive stimuli such as drugs of abuse significantly increase extracellular dopamine in the NAc and PFC, but the dorsal striatum

appears to be acutely less sensitive to such stimuli (Bassareo, Tanda, Petromilli, Giua, & Di Chiara, 1996; Cenci, Kalén, Mandel, & Björklund, 1992; Di Chiara & Imperato, 1988; Horvitz, 2000; Howard et al., 2008; Mark, Smith, Rada, & Hoebel, 1994; Schier et al., 2013; Valenta et al., 2013; Willuhn et al., 2012). Aversive and stressful stimuli have been shown to increase extracellular dopamine in the PFC to a much greater extent than in the NAc or dorsal striatum (Abercrombie et al., 1989; Bassareo et al., 1996; Cenci et al., 1992; Horvitz, 2000; Kalivas & Duffy, 1995; Sorg & Kalivas, 1993b). Additionally, aversive stimuli increase the AMPAR/NMDAR ratio only in dopaminergic cells projecting to the PFC and lateral NAc shell, indicating modulation of excitatory synapses on these subpopulations of dopamine neurons (Lammel, Ion, Roeper, & Malenka, 2011). In contrast, AMPAR/NMDAR ratios increased only in those dopamine neurons projecting to medial and lateral NAc in response to acute cocaine reward (Lammel et al., 2011, 2014). Similarly, rats exposed to a single high dose of toluene vapor demonstrated significant increases in AMPA/NMDA ratios in VTA dopamine neurons projecting to the NAc core and medial shell, but not in mesocortical dopamine neurons (Beckley, Evins, Fedarovich, Gilstrap, & Woodward, 2013). Therefore, subpopulations of midbrain dopamine neurons appear serve distinct roles in the response to salient events depending on the motivational valence of the event (for reviews see (Marinelli & McCutcheon, 2014; Volman et al., 2013)), and this may have functional relevance to

stimulation of dopamine activity observed in specific target regions following acute ethanol administration

While the cellular and molecular mechanisms by which ethanol stimulates mesocorticolimbic dopamine activity are not entirely understood, ethanol may potentially exert differential effects on midbrain dopamine neuron subpopulations. Therefore, the anatomical distribution and physiological, molecular, and functional heterogeneity of midbrain dopamine neurons may contribute to the regional differences observed in the “response ratio” analyses. Ethanol has been shown to directly stimulate VTA dopamine neurons (Brodie et al., 1999, 1990), but the projection targets of the recorded neurons were not identified. Differential effects of ethanol have been observed in the VTA with respect to the anterior and posterior regions. Rats will self-administer various doses of ethanol directly into the posterior VTA, but not the anterior VTA (Rodd-Henricks, McKinzie, Crile, Murphy, & McBride, 2000). Recently, ethanol has been shown to increase the firing rate of dopamine neurons located in the posterior VTA, but suppresses the firing rate of dopamine neurons originating in the anterior VTA (Guan et al., 2012). These differential effects of ethanol on the anatomical divisions of the VTA may contribute to the differences seen in the “response ratios” in target regions. The dopamine neurons projecting to the PFC, NAc core and medial shell, and BLA form distinct populations within the medial posterior VTA (Lammel et al., 2008, 2014). In contrast, dopamine neurons projecting to the lateral NAc shell

are found in the lateral posterior and anterior VTA, with a significant number of these neurons also located in the SNc (Lammel et al., 2008).

Additionally, acute ethanol may selectively modulate excitatory (and/or inhibitory) synapses on VTA dopamine neurons, similar to the effect observed following acute cocaine or toluene administration (Beckley et al., 2013; Lammel et al., 2011). Acute systemic administration of ethanol has been shown to strengthen excitatory synapses on VTA dopamine neurons, as indicated by increased AMPAR/NMDAR ratios (Saal, Dong, Bonci, & Malenka, 2003), but because the projection targets of these neurons were not identified, it is unclear if this effect uniform across dopamine neurons. Ethanol may exert differential effects on midbrain dopamine neurons, such as selectively enhancing firing rates or excitatory/inhibitory synapses, which could alter dopamine activity in target regions and thus, potentially contribute to the regional differences in “response ratios”.

Regional differences in dopamine clearance

The observed regional differences in the temporal profiles of the “response ratios” may be due, at least in part, to regional differences in the mechanisms of dopamine clearance or variations in the sensitivity of the clearance mechanisms to ethanol. Early on it was demonstrated that regional differences exist in the dynamic regulation of extracellular dopamine. Garris and

Wightman determined ratios of dopamine release to uptake to quantify and compare the regulation of extracellular dopamine across the PFC and striatal regions (Garris & Wightman, 1994). In striatal regions, this ratio is low, indicating "uptake-dominant" regulation of extracellular dopamine concentrations. In contrast, this ratio is 5-10 times larger in the PFC, indicating "release-dominant" dynamics of interstitial dopamine. Furthermore, dopamine terminals in the PFC show a reduced density of dopamine transporters relative to striatal regions (Cass & Gerhardt, 1995; Javitch, Strittmatter, & Snyder, 1985).

Clearance of evoked dopamine in the PFC appears slower than that in the striatum and uptake by high affinity dopamine transporters (DAT) is not the primary mechanism of clearance (Cass & Gerhardt, 1995; Yavich, Forsberg, Karayiorgou, Gogos, & Männistö, 2007). Studies comparing the effect of DAT blockade across brain regions consistently demonstrate reduced efficacy of DAT inhibition on extracellular dopamine in the PFC relative to striatal regions (Budygin et al., 1999; Cass & Gerhardt, 1995; Käenmäki et al., 2010; Mazei, Pluto, Kirkbride, & Pehek, 2002; Wayment, Schenk, & Sorg, 2001). For example, the dopamine uptake inhibitor GBR-12909 increases the amplitude and time course of dopamine signals by 200% in the striatum, which contrasts with the 30-40% increase in these parameters observed in the PFC (Cass & Gerhardt, 1995; Izenwasser, Werling, & Cox, 1990).

Other work has focused on the predominant role of metabolism relative to catecholamine uptake mechanisms on dopamine clearance in the PFC. Using *in*

vitro voltammetry, Wayment et al. demonstrated a linear rate of clearance in the PFC, but pharmacological blockade of DAT/NET (norepinephrine transporter) and inhibition of monoamine oxidase (MAO) produced a biphasic dopamine clearance profile due to an additive effect of the drugs. Based on these findings, Wayment and colleagues concluded that dopamine clearance velocity in the PFC is 50-70% dependent on uptake mechanisms (DAT/NET) and 30-50% dependent on MAO (Wayment et al., 2001). However, this study did not address the role of metabolism by catechol-O-methyltransferase (COMT), which is particularly important for dopamine clearance in regions where DAT density is low and has been demonstrated to play a significant role in dopamine clearance in the PFC (Käenmäki et al., 2010; Schott et al., 2010; Tunbridge, Bannerman, Sharp, & Harrison, 2004; Yavich et al., 2007). COMT mRNA expression is significantly higher in the PFC than the striatum in human and rat brains (Matsumoto et al., 2003). COMT metabolizes dopamine to 3-methoxytyramine (3-MT), which accounts for approximately 60% of the total dopamine turnover in the frontal cortex, but only 15% in the striatum (Karoum, Chrapusta, & Egan, 1994). Additionally, pharmacological inhibition of COMT by tolcapone in the PFC significantly increases evoked extracellular dopamine (Lapish et al., 2009; Tunbridge et al., 2004). In contrast, systemic administration of tolcapone does not alter extracellular dopamine in the striatum, except under the conditions of dopamine uptake inhibition (Budygin et al., 1999). In summary, dopamine clearance in the PFC relies heavily on metabolism, while in striatal regions

dopamine clearance is driven by reuptake mechanisms. If the rate of decline of the dopamine signal is differentially regulated across brain regions, then this could be a potential explanation for the observed regional differences in the temporal profiles of the “response ratios”.

Examination of the interaction between ethanol and dopamine clearance mechanisms has predominantly focused on DAT. Acute ethanol administration has been shown to enhance (Mayfield, Maiya, Keller, & Zahniser, 2001; Y. Wang, Palmer, Cline, & Gerhardt, 1997), decrease (Robinson, Volz, Schenk, & Wightman, 2005), or not affect (Budygin et al., 2001; Yim & Gonzales, 2000) DAT uptake velocity in the striatum. Of note, however, is that despite the discrepant observations of ethanol’s effects on DAT activity, there appears to be agreement that ethanol does not alter the transporter’s affinity for dopamine (Mayfield et al., 2001; Robinson et al., 2005). Genetic manipulations may provide a means of resolving the discrepant results. DAT-knockout (DAT-KO) mice show similar increases in extracellular dopamine in the dorsal striatum as wild type (WT) mice following acute systemic administration of ethanol, which is consistent with previous work demonstrating that direct inhibition or reduction in DAT activity by ethanol is not a primary mechanism underlying stimulation of striatal dopamine activity (Mathews, John, Lapa, Budygin, & Jones, 2006). Furthermore, fast-scan cyclic voltammetry in brain slices from DAT-KO and WT mice demonstrated no effect of 20 or 200 mM ethanol on the rate of dopamine clearance in the dorsal

striatum (Mathews et al., 2006). However, to date there are no published studies exploring the effect of acute ethanol on DAT in the PFC.

At this time, limited work has explored the interaction between acute ethanol and dopaminergic metabolic mechanisms in the PFC. While early studies demonstrated increased tissue concentrations of dopamine metabolites in the striatum and PFC of animals that received acute systemic ethanol administration, it is unclear if these elevations are a direct result of ethanol-induced increases in extracellular dopamine or if these effects vary depending on the ethanol dose (Fadda, Argiolas, Melis, Serra, & Gessa, 1980; Milio & Hadfield, 1992; Reggiani, Barbaccia, Spano, & Trabucchi, 1980). Further research is necessary to determine if ethanol directly affects the activity of enzymes involved in dopamine metabolism, specifically within the PFC, as these enzymes may be potential therapeutic targets in alcohol use disorders.

CONCLUSION

In conclusion, a dissociation exists in the temporal profiles of extracellular concentrations of dopamine and tissue concentrations of ethanol, which may be attributable to ethanol's mechanism of action. Within the first 25-30 minutes following acute i.v. ethanol administration the time course of this dissociation demonstrates regional variability. Such variability may be due to ethanol's pharmacological interactions with a heterogeneous population of midbrain

dopamine neurons, regional differences in dopamine clearance mechanisms, and/or acute modulation of dopamine clearance mechanisms by ethanol. Further investigation is necessary to determine if ethanol exerts such effects on dopamine activity, the precise cellular and molecular mechanisms by which ethanol enhances mesocorticolimbic dopamine activity, and if the ethanol-induced transient rise and decline in extracellular dopamine contributes to the development of acute tolerance to the stimulating effects of ethanol.

Chapter 3:

Regional analysis of the pharmacological effects of acute ethanol on extracellular striatal dopamine activity

The work presented in this chapter will be published in December 2016 in Alcoholism: Clinical and Experimental Research by Vena, Mangieri, and Gonzales. This publication has been reprinted with permissions from John Wiley & Sons (License #: 3994840327842). Ashley Vena contributed to data collection and analyses, and prepared and submitted the final manuscript.

ABSTRACT

The objective of the present study was to characterize the acute pharmacological effects of ethanol on extracellular dopamine in the dorsomedial and dorsolateral striata. This is the first study to quantify and directly compare the effects of acute ethanol on dopamine in these subregions. Therefore, we also tested the nucleus accumbens as a positive control. We hypothesized that while ethanol may increase extracellular dopamine in the dorsomedial striatum and dorsolateral striatum, the magnitude of this increase and the temporal profiles of extracellular dopamine concentrations would differ among the dorsomedial striatum, dorsolateral striatum, and nucleus accumbens. We performed in vivo microdialysis in adult, male Long Evans rats as they received a single (experiment 1) or repeated (experiment 2) doses of ethanol. The results of our positive control study validate earlier work by our lab demonstrating that acute

intravenous ethanol immediately and robustly increases extracellular dopamine in the nucleus accumbens (Howard et al., 2008). In contrast, a single 1 g/kg dose of intravenous ethanol did not significantly affect extracellular dopamine in the dorsomedial striatum or the dorsolateral striatum. However, following a cumulative ethanol dosing protocol we observed a ramping up of tonic dopamine activity in both the dorsomedial striatum and dorsolateral striatum over the course of the experiment, but this effect was more robust in the dorsomedial striatum. These results suggest that distinct mechanisms underlie the stimulating effects of acute ethanol on extracellular dopamine in striatal subregions. Additionally, our findings suggest a role for the dorsomedial striatum and minimal to no role for the dorsolateral striatum in mediating the intoxicating effects of acute moderate to high doses of ethanol.

INTRODUCTION

Several studies have demonstrated that the mesolimbic and nigrostriatal dopaminergic systems are sensitive to the pharmacological effects of acute ethanol. In vitro electrophysiological studies by Brodie et al. (1999,1990) demonstrated that ethanol dose-dependently increases the firing rate of dopamine cell bodies in the ventral tegmental area. Mereu et al. (1984) reported that in paralyzed rats, intravenous administration of low to moderate doses of ethanol (0.5-2.5 g/kg) increased the firing rate of dopamine cell bodies in the substantia nigra, while high doses (4.0 g/kg and higher) suppressed firing rates.

In vivo neurochemical studies sampling from striatal regions innervated by ventral tegmental area dopamine neurons confirm an effect of acute ethanol on mesolimbic dopamine activity, suggesting a dose-dependent, biphasic effect of ethanol on extracellular dopamine activity, Microdialysis and voltammetry studies indicate that low to moderate doses of systemic ethanol (0.5-2.5 g/kg) increase extracellular dopamine in the nucleus accumbens (Robinson et al. 2009; Howard et al. 2008; Yim et al. 2000a; Imperato & Di Chiara 1986). Higher doses of ethanol (5 g/kg), however, appear to depress extracellular dopamine activity in the nucleus accumbens (Imperato & Di Chiara, 1986).

Relative to the mesolimbic dopamine system, in vivo microdialysis studies exploring the effects of acute ethanol on nigrostriatal dopamine activity are less consistent. Imperato & Di Chiara (1986) demonstrated that low doses of systemic ethanol (0.25-0.5 g/kg; i.p.) had no significant effect on dorsal striatal dopamine activity, while moderate to high doses (1.0-5.0 g/kg; i.p.) increased extracellular dopamine relative to baseline. In contrast, Blanchard et al. (1993) reported enhanced extracellular dopamine following low doses of ethanol (i.p.) and no effect or decreases following moderate to high doses of ethanol (i.p.). Despite these inconsistent findings, it appears that substantia nigra dopamine neurons are generally less sensitive to acute ethanol as any increases from baseline are less robust in the dorsal striatum relative to the nucleus accumbens.

A potential limitation of these earlier studies is that the dorsal striatal subregions were not tested separately. The dorsomedial and dorsolateral striata

have been shown to be distinct functionally and in the origins of their dopamine afferents (Joel & Weiner 2000; Yin et al. 2008; Voorn et al. 2004). The dorsomedial striatum is critical for learning the action-outcome associations that underlie goal-directed behaviors, while the dorsolateral striatum is critical for learning the stimulus-response associations that underlie habit formation (Yin et al. 2008). Dopamine afferents to the dorsomedial striatum originate in the retrorubral area, substantia nigra pars compacta and the ventral tegmental area (Joel & Weiner, 2000; Voorn et al., 2004). In contrast, the dorsolateral striatum only receives dopaminergic projections from the substantia nigra and retrorubral area (Voorn et al. 2004; Joel & Weiner 2000). Recent work has demonstrated heterogeneity in the sensitivity of midbrain dopamine neurons to drugs of abuse, including ethanol (Lammel et al., 2011; Mrejeru, Martí-Prats, Avegno, Harrison, & Sulzer, 2015). Therefore, it is possible that midbrain dopamine projections to the dorsomedial striatum and dorsolateral striatum may differ in their sensitivity to ethanol, which may be assessed by measuring extracellular dopamine concentrations in these target subregions.

In this study we sought to characterize and directly compare the acute pharmacological effects of ethanol on extracellular dopamine in both the dorsomedial striatum and dorsolateral striatum using microdialysis in awake, freely moving animals. Intravenous administration of either a single dose (1 g/kg) or a cumulative dosing procedure was used to minimize the effects of stress and handling on extracellular dopamine activity, as well as to avoid the potentially

confounding effects of behavior, expectation, and motivation on dopamine that may be evident in a self-administration design. Our results indicate that dopamine in these subregions is differentially affected by ethanol.

METHODS

Animals

A total of fifty-three adult Long Evans rats were used in these experiments. Twenty-six rats (from Charles River, Raleigh, NC), weighing 300-430 grams on dialysis day, were used for the acute ethanol experiments. Twenty-seven rats (from Harlan, Indianapolis, IN), weighing 310-450 grams on dialysis day, were used for the cumulative dose-response experiments. Rats were housed in a temperature (25° C) and light (12 hours on/12 hours off) controlled room and had access to chow and tap water ad libitum. Upon arrival, the rats were dually-housed for the first week, during which they were handled and weighed daily. All procedures were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Surgery

Cannulation and jugular catheterization surgeries were carried out according to the procedures described in Duvauchelle (1998) and Howard et al.

(2008). Intravenous catheters were constructed from silastic tubing (0.30 mm ID, 0.64 mm OD, Fisher Scientific, Hampton, NH), a metal cannula (22 gauge, Plastics One, Roanoke, VA), and silicone adhesive. Rats were anesthetized with isoflurane and an incision was made above the skull. Upon securing the catheter in the jugular vein, it was pulled subcutaneously to the top of the skull. A microdialysis guide cannula (21 gauge, Plastics One, Roanoke, VA) was implanted into the skull directly above the nucleus accumbens (AP +2.0, ML +1.1, DV -3.4), dorsomedial striatum (AP +1.2, ML +1.8, DV -1.0), or the dorsolateral striatum (AP 0.0, ML +3.7, DV -1.0) while the animal was in a stereotaxic frame. The guide cannula, catheter cannula, and a tether bolt were held in place on the skull with dental cement. A Timentin (13.4 mg/kg, Animal Health International) and heparinized saline solution was used as a catheter lock solution to maintain patency. Catheters were flushed with 0.2 mL of heparinized saline at least once a week before dialysis experiments commenced. Following surgeries, rats were individually housed and given at least 6 days of recovery prior to experiments.

Drugs

A 10 % (w/v) solution of ethanol (1 g/kg, 10 ml/kg) in saline was made from 95 % ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY).

Microdialysis

Approximately 12-18 hours prior to the microdialysis experiment, rats were lightly anesthetized with isoflurane to implant the microdialysis probe through the guide cannula and to secure the animal to the tethering apparatus. The probes (1.5 mm active membrane length, 270 μm OD, 13,000 MWCO) were constructed in the laboratory according to the procedures described by Pettit & Justice (1991). Probes were continuously perfused with artificial cerebrospinal fluid (ACSF; 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.2 mM ascorbic acid, and 5.4 mM D-glucose) overnight at 0.2 μL/min. The flow rates were increased to 2.0 μL/min at least 2 hours prior to dialysate sample collection and remained at 2.0 μL/min for the duration of the experiment.

Animals were awake and freely moving during the microdialysis experiments. In all experiments, the sample collection interval was 5 minutes and four baseline samples were collected per animal prior to any infusions. Baseline dopamine concentrations were required to have a relative standard deviation <0.16 for data inclusion. To confirm that the dopamine in the dialysate samples was due to calcium-dependent exocytotic release, probes were perfused with calcium-free ACSF for approximately 2 hours at the conclusion of all experiments and additional samples were collected. All dialysate samples were immediately frozen on dry ice upon collection.

Experiment 1: Acute intravenous 1 g/kg ethanol

Following collection of the baseline samples, animals received a manual bolus of saline. Samples were collected for 45 minutes following the infusion. Animals then received a manual bolus of a 1 g/kg dose of ethanol (10% w/v, in saline), and post-infusion samples were collected for 25 minutes (Table 1).

Experiment 2: Cumulative intravenous ethanol

For the cumulative dosing study, animals received 4 infusions of ethanol (10% w/v, in saline) or saline. Infusions were administered 20 minutes apart via a syringe pump (CMA 400, Japan) at a flow rate of 1 mL/min. All animals received a saline infusion at least 1 hour prior to sample collection to habituate them to the sound of the pump. Ethanol infusions were administered in the following order: 0.5, 0.5, 0.75, and 0.75 g/kg (Table 1). This produced cumulative doses of 0.5, 1.0, 1.75, and 2.25 g/kg.

Table 1: Timeline of microdialysis experiments

Experiment 1	
Sample	**Experimental phase**
1-4	Baseline samples
5	Saline infusion
6-9	Post-saline infusion
10-13	Baseline samples
14	Ethanol infusion (1 g/kg)
15-18	Post-ethanol infusion

Experiment 2	
Sample	**Experimental phase**
1-4	Baseline samples
5	Ethanol (0.5 g/kg) or saline infusion
6-8	Post infusion 1
9	Ethanol (0.5 g/kg) or saline infusion
10-12	Post infusion 2
13	Ethanol (0.75 g/kg) or saline infusion
14-16	Post infusion 3
17	Ethanol (0.75 g/kg) or saline infusion
18-20	Post infusion 4

Histology

After the dialysis experiments, rats were overdosed with sodium pentobarbital (150 mg/kg, i.v.) and probes were carefully unimplanted. Their brains were harvested and stored in vials containing 10% formalin for at least 24 hours. The brain tissue was coronally sectioned (120 μm thick), stained with cresyl violet, and examined under a microscope to confirm probe placement. The probe tracks were mapped using the Paxinos & Watson (2007) atlas.

HPLC Analysis

Dialysate dopamine concentrations were quantified via reversed-phase high performance liquid chromatography with electrochemical detection. The HPLC systems consisted of a Luna 50 x 1.0 mm column (C18, 3 μm particle size; Phenomenex, Torrance, CA), 2 mm glassy carbon working electrode electrochemical detector (SenCell; Antec Leyden) at potential +450 mV, an 8125 manual injector (Rheodyne, Cotati, CA), and an INTRO controller (Antec Leyden). Mobile phase was continuously pumped through the systems via either a syringe pump (ISCO 65D, Telodyne) or an LC110S pump (Antec Leyden). The mobile phase consisted of 0.500 g octanesulfonic acid, 0.050 g decanesulfonic acid, 0.128 g ethylenediaminetetraacetic acid, and 11.08 g NaH_2PO_4 dissolved in 1 liter of deionized water, and methanol as the organic solvent (8-10% v/v). The mobile phase was adjusted to pH 5.6 prior to adding the methanol. The sample

injection volume was 5 microliters. External standards (0.3125 to 7.5 nM) were used to quantify the dopamine concentrations. EZChrom Elite software (Agilent, Wilmington, DE) was used to record and analyze all chromatograms. Only dopamine peaks with a signal to noise ratio >6 were included in the analyses.

GC Analysis

For animals that received ethanol infusions, dialysate ethanol concentrations were quantified via gas chromatography (GC) with flame ionization detection. Prior to freezing the dialysate samples, 2 µL aliquots were transferred to 2 mL glass chromatography vials and sealed with a septum. A Varian CP 3800 (Agilent Technologies) or Scion 436 gas chromatograph (Bruker, Netherlands) and a Varian 8200 headspace autosampler was used to analyze the concentrations of ethanol in the samples. The stationary phase was an HP Innowax capillary column (30 m × 0.53 mm × 1.0 µm film thickness). For the acute, single-dose studies, helium was used as the carrier gas, but prior to the cumulative dosing studies a hydrogen generator (Model 20H-MD, Parker Hannifin, England) was installed to use hydrogen as the carrier. Resulting ethanol peaks were recorded using either Varian Star Chromatography Workstation or CompassCDS (Bruker, Netherlands) software, and calibration was achieved using external standards (0.3125 to 40 mM).

Statistical analyses

Repeated measures analyses of variance (ANOVA) were performed on both the dialysate dopamine concentrations and the dialysate dopamine values normalized to baseline. Repeated measures ANOVAs were also performed on the dialysate ethanol concentrations. Two animals from experiment 1 (1 each from the nucleus accumbens and dorsolateral striatum groups) were missing 1-2 dialysate ethanol values due to technical issues with the GC. In order to have the animals included in statistical analyses, the mean of the surrounding values was used in place of the missing value. For the overall repeated measures ANOVA on the dialysate ethanol data, the degrees of freedom were appropriately adjusted to account for these missing values.

In experiment 1, we analyzed the effects of the saline and ethanol infusions separately, using 2-way mixed-model, repeated measures ANOVAs. Separate ANOVAs were performed on the first 9 time points (4 baseline and 5 post-saline infusion) and the second 9 time points (4 baseline and 5 post-ethanol infusion). The four samples prior to each infusion were used to determine basal dopamine concentrations for each animal. In each ANOVA, the between subjects factor was brain region (3 levels: nucleus accumbens, dorsomedial striatum, dorsolateral striatum), and the within subjects factor was time.

In experiment 2, we performed a three-way, mixed model, repeated measures ANOVA. The 2 between-subjects factors were brain region (2 levels: dorsomedial striatum, dorsolateral striatum) and drug (2 levels: saline, ethanol) and the within-subjects factor was time (20 time points). Additionally, because

our a priori hypothesis was that cumulative infusions of ethanol would differentially affect extracellular dopamine within the dorsomedial and dorsolateral striata relative to cumulative saline infusions, we also explored the time by drug interaction within each brain region. Samples 1-4 were used to determine basal dopamine concentrations for each animal. Sample 10 from an animal in the dorsomedial striatum-saline group was contaminated and thus removed from the dataset. The missing value was replaced with the mean of the surrounding samples and the degrees of freedom were appropriately adjusted.

We performed simple effects analyses (with Bonferroni corrections) when appropriate to follow up on any significant interaction effects identified in the overall ANOVAs (Kirk, 1982). Data were analyzed using SPSS software (IBM). Significance was assigned if $p < 0.05$; NS = not significant.

Table 2: Basal dialysate dopamine concentrations and number of animals included in analyses for each region

	Experiment 1				**Experiment 2**			
	Saline Infusion		**Ethanol Infusion***		**Saline Group**		**Ethanol Group**	
Region	***n***	**[DA] (nM)**	***n***	**[DA] (nM)**	***n***	**[DA] (nM)**	***n***	**[DA] (nM)**
DMS	9	1.8 ± 0.1	7	1.8 ± 0.1	5	1.5 ± 0.3	8	2.0 ± 0.2
DLS	7	1.3 ± 0.2	6	1.3 ± 0.2	7	1.7 ± 0.2	7	2.3 ± 0.3
Nac	5	1.3 ± 0.3	8	0.9 ± 0.1	n/a		n/a	

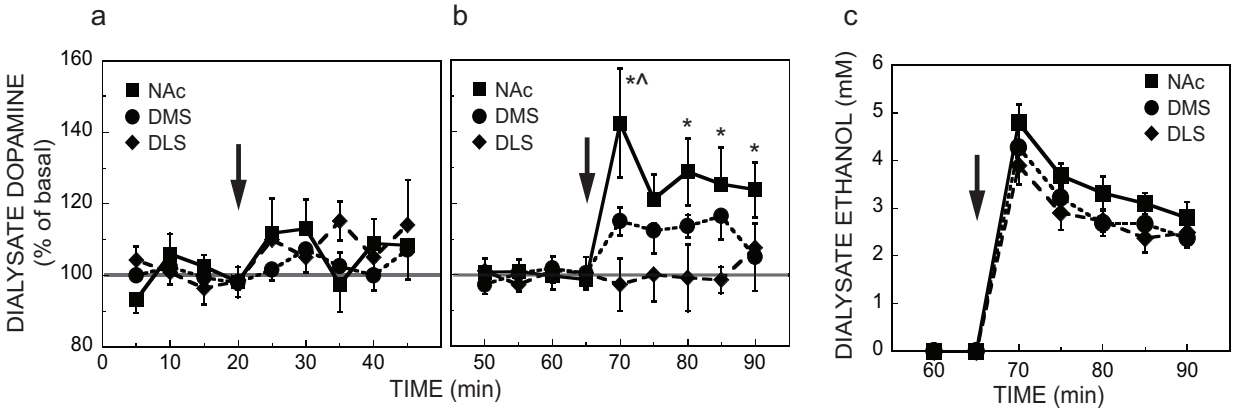
Basal dopamine concentrations for each brain region (mean ± SEM). In experiment 1, separate baseline samples were collected prior to both the saline and ethanol infusions. In experiment 2, basal dopamine concentrations were determined from the first 4 dialysate samples. The asterisk (*) indicates significance difference in basal values across brain regions ($p<0.05$).

RESULTS

Basal dopamine concentrations

Basal dopamine concentrations for each brain region are reported in Table 1. In experiment 1, the saline and ethanol baseline values for each animal were analyzed separately. Therefore, it was possible for an animal's saline data to be excluded (due to instability of the baseline values) despite the ethanol data meeting all inclusion criteria, and vice versa. Due to significant differences in baseline dopamine concentrations among the 3 brain regions prior to the ethanol infusion ($F_{2,18}$=12.05, $p<0.01$), we did the statistical analyses on the percent basal data for experiment 1. For experiment 2, because there were no significant differences in basal dopamine values, we analyzed the nanomolar dialysate dopamine concentrations.

Figure 7: Dialysate dopamine in the DMS, DLS, and NAc (percent of baseline)



Dialysate dopamine in the DMS, DLS, and NAc represented as a percent of basal levels following intravenous administration of (a) saline (0.9% NaCl) and (b) ethanol (1 g/kg). Figure (c) shows the temporal profiles of dialysate ethanol concentrations in the DMS, DLS, and NAc. Symbols represent the mean. Error bars are SEM, but not all are shown for clarity. Asterisk (*) indicates significant difference from baseline ($p<0.05$; nucleus accumbens only), and the caret (^) indicates significant differences among the three brain regions at that time point ($p<0.01$; Bonferroni). The arrow indicates the time of the infusions.

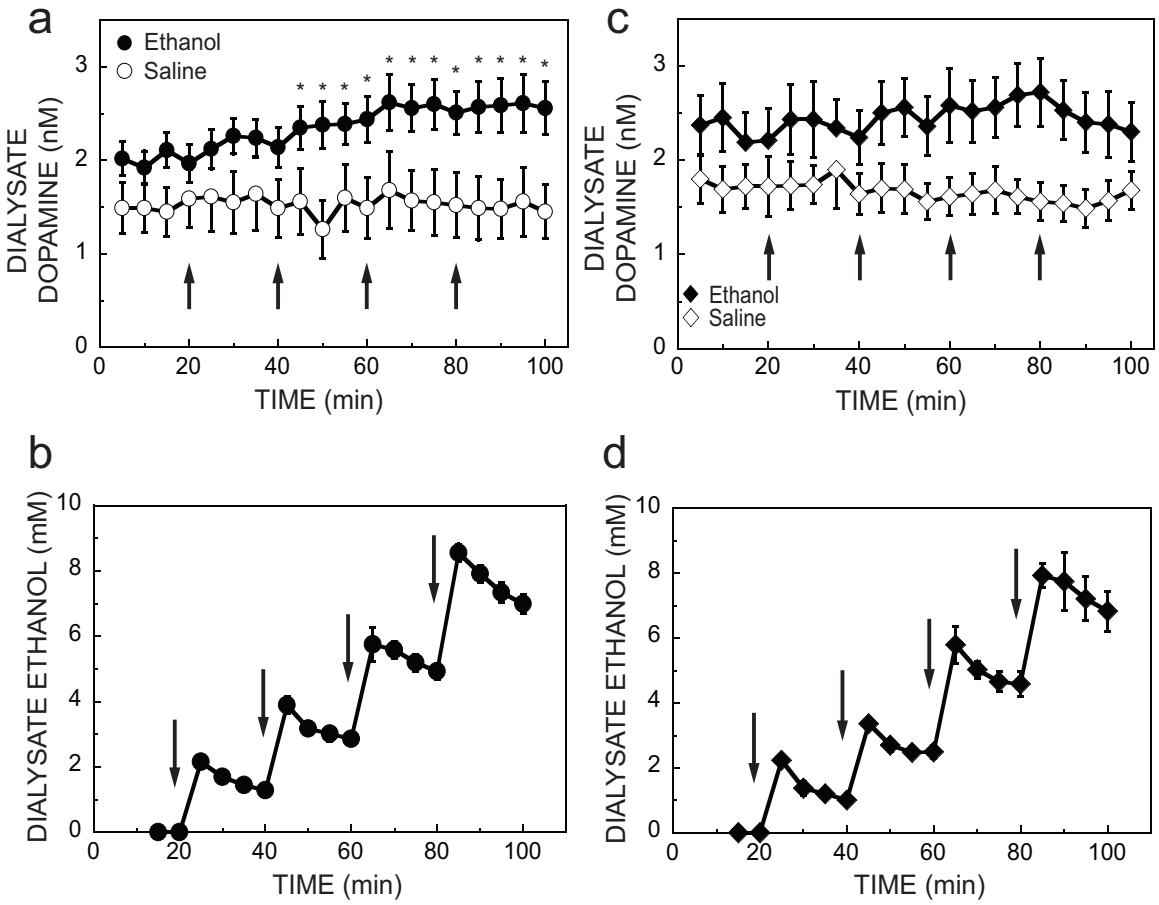
Single dose 1 g/kg

Control infusions of saline had no significant effect on dopamine activity in any subregion (Fig 7a; $F_{8,144}$=1.89, NS). In contrast, the ethanol infusion stimulated dopamine release in some but not all striatal subregions (subregion x time interaction: $F_{16,144}$=2.06, $p<0.05$). In the nucleus accumbens, extracellular dopamine was significantly elevated above baseline following the ethanol infusion (n=8; Fig 7b; simple effect of time: $F_{8,144}$=7.98, $p<0.01$). Post hoc analyses showed that ethanol-induced stimulation of dopamine peaked at 40% above baseline within the first 5 minutes following the infusion ($F_{4,144}$=11.16, $p<0.01$). Furthermore, dopamine remained elevated in the nucleus accumbens 15-25 minutes following the infusion ($F_{4,144}$=3.54-5.14, $p<0.01$ for each time point). In the dorsomedial striatum, the same dose of ethanol stimulated dopamine to 16% above baseline, but this effect was not statistically significant (n=7; Fig 7b; simple effect of time: $F_{8,144}$=1.49, NS). In the dorsolateral striatum, there was clearly no effect of ethanol on extracellular dopamine (n=6; Fig 7b; simple effect of time: $F_{8,144}$=0.22, NS).

Simple effect analysis indicated that the dopamine response in the sample immediately following the ethanol infusion differed among the three brain regions ($F_{2,138}$=11.5, $p<0.001$). Post hoc tests found that the response in the nucleus accumbens was significantly greater than that in the dorsomedial striatum ($F_{1,138}$=8.84, $p<0.01$) and dorsolateral striatum ($F_{1,138}$=22.04, $p<0.01$). However, extracellular dopamine concentrations in the sample immediately following the

ethanol infusion were not significantly different between the dorsomedial and dorsolateral striatum ($F_{1,138}$=3.21, NS). Additional simple effects and post hoc analyses revealed only significant differences between the nucleus accumbens and the dorsolateral striatum ($F_{1,138}$=9.44, $p<0.01$) at the 80-minute time point (15 minutes following the ethanol infusion). Lastly, the dialysate ethanol time courses were not significantly different among the 3 subregions (Fig 7c; $F_{2,18}$=1.76, NS).

Figure 8: Temporal profiles of dialysate dopamine (nM) and ethanol concentrations (mM)



Temporal profiles of dialysate dopamine (nM) and ethanol concentrations (mM) for DMS (a and b, respectively) and DLS (c and d, respectively) following cumulative intravenous infusions. Separate groups of rats received either ethanol doses (0.5, 0.5, 0.75, and 0.75 g/kg; filled symbols) or control saline infusions (open symbols). Symbols represent the mean. Error bars are SEM, but not all are shown for clarity. The arrow indicates the time of the infusion.

Cumulative dose-response

The objective of Experiment 2 was to use a cumulative-dosing design to test whether a large ethanol dose range had differential effects on extracellular dopamine in the dorsomedial striatum and dorsolateral striatum. There were 4 groups: dorsomedial striatum-ethanol, dorsomedial striatum-saline, dorsolateral striatum-ethanol, and dorsolateral striatum-saline. Within each group, the within-subjects factor was time. Infusions occurred at the 20, 40, 60, and 80-minute time points.

The overall ANOVA conducted on the raw (untransformed; Figs 8a and 8c) nanomolar dopamine concentrations indicated significant main effects of time (within-subjects; $F_{19,436}=3.02$, $p<0.001$) and drug (between-subjects; $F_{1,23}=8.28$, $p<0.01$) on extracellular dopamine. While the three-way interaction between time, drug, and brain region was not significant ($F_{19,436}=0.94$, NS), there were significant interactions of time by drug ($F_{19,436}=5.08$, $p<0.001$) and of time by brain region ($F_{19,436}=2.08$, $p<0.01$). Post-hoc analyses following up on the interaction of time and drug indicated a significant simple effect of time only for the ethanol groups ($F_{19,436}=8.43$, $p<0.001$; collapsed across dorsomedial striatum and dorsolateral striatum). Cumulative saline infusions had no significant effect on extracellular dopamine concentrations ($F_{19,436}=1.04$, NS; collapsed across dorsolateral striatum and dorsomedial striatum). Additional post hocs examining drug effects indicated significant differences between the saline and ethanol

groups at time points 55-90 minutes ($F_{1,28}$=5.40, p<0.01; collapsed across dorsomedial striatum and dorsolateral striatum).

Following up on the time by brain region interaction, post hoc analyses indicated a significant simple effect of time on extracellular dopamine only in the dorsomedial striatum ($F_{19,436}$=5.40, p<0.001; collapsed across saline and ethanol groups). However, subsequent post hoc analyses comparing baseline vs individual time points were not statistically significant (Fig 8a). The effect of time was not significant for the dorsolateral striatum ($F_{19,436}$=1.38, NS; collapsed across saline and ethanol groups). Additionally, because our a priori hypothesis was that ethanol would differentially affect dopamine concentrations in the dorsomedial and dorsolateral striatum, we also analyzed drug effects within each subregion. A significant time by drug interaction was observed in both the dorsomedial ($F_{19,436}$=3.26, p<0.001) and dorsolateral ($F_{19,436}$=2.71, p<0.001) striatum. However, subsequent post hoc analyses comparing ethanol time points vs. baseline or ethanol vs. saline at individual time points were not statistically significant for either subregion.

Separate analyses were performed on the normalized data (Figure 9). Similar to the results from the analyses on the raw data, the overall ANOVA indicated significant main effects of time (within-subjects; $F_{19,436}$=2.93, p<0.001) and drug (between-subjects; $F_{1,23}$=10.84, p<0.01), as well as a significant interaction of time by drug ($F_{19,436}$=10.58, p<0.001). The three-way interaction between time, drug, and brain region was not significant ($F_{19,436}$=0.90, NS), but

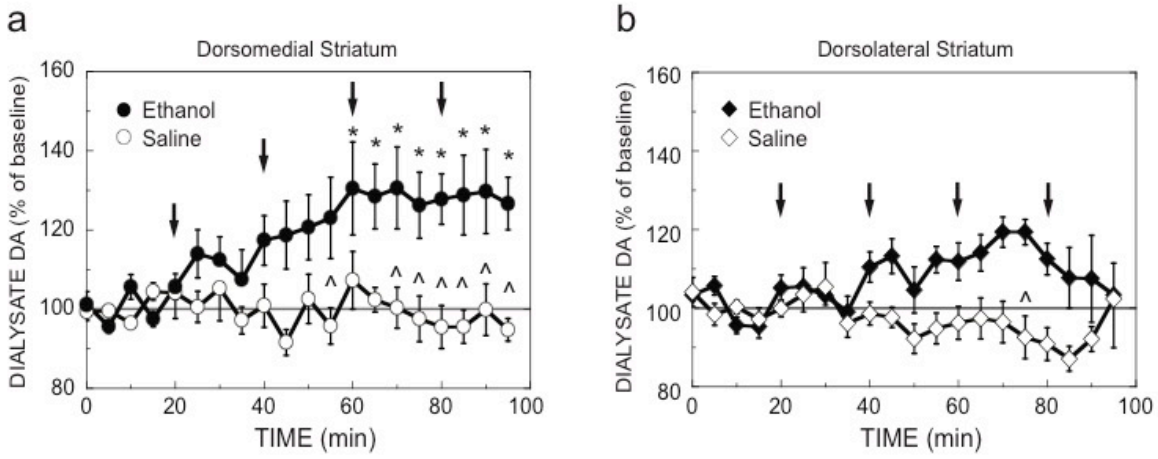
the two way interaction between time and brain region trended towards significance ($F_{19,436}$=1.46, p=0.097). Post hoc analyses following up on the overall time by drug interaction indicated a significant simple effect for the ethanol groups only ($F_{19,436}$=8.57, p<0.001; collapsed across dorsomedial and dorsolateral striatum). Cumulative saline infusions had no significant effect on extracellular dopamine ($F_{19,436}$=1.09, NS; collapsed across dorsomedial and dorsolateral striatum).

Although the interaction between time and brain region did not meet the criteria for statistical significance in our analyses of the normalized dopamine data, our a priori hypothesis was that ethanol would exert differential effects on dopamine based on subregion. Therefore, we analyzed drug and time effects within each brain region. A significant time by drug interaction was observed in both the dorsomedial striatum (Fig 9a; $F_{19,436}$=3.77, p<0.001) and dorsolateral striatum (Fig 9b; $F_{19,436}$=2.39, p<0.001). Additionally, the simple effect of time was significant in both the dorsomedial striatum ($F_{19,436}$=8.17, p<0.001) and dorsolateral striatum ($F_{19,436}$=2.38, p<0.001), only for the ethanol groups and not the saline groups. However, subsequent post hoc analyses comparing ethanol time points vs. baseline or ethanol vs. saline at individual time points were statistically significant only for the dorsomedial striatum (Fig 9a).

Between the dorsomedial striatum and the dorsolateral striatum, there were no significant differences in the temporal profiles of the dialysate ethanol concentrations (Figs 8b and 8d, respectively; $F_{1,13}$=0.92, NS). Dialysate ethanol

concentrations increased with each ethanol infusion, peaking at 8.5 ± 0.3 mM in the dorsomedial striatum and 7.9 ± 0.4 mM in the dorsolateral striatum.

Figure 9: Dose-response effect of cumulative infusions of ethanol and saline on dialysate dopamine in the dorsomedial striatum and the dorsolateral striatum (percent of baseline)

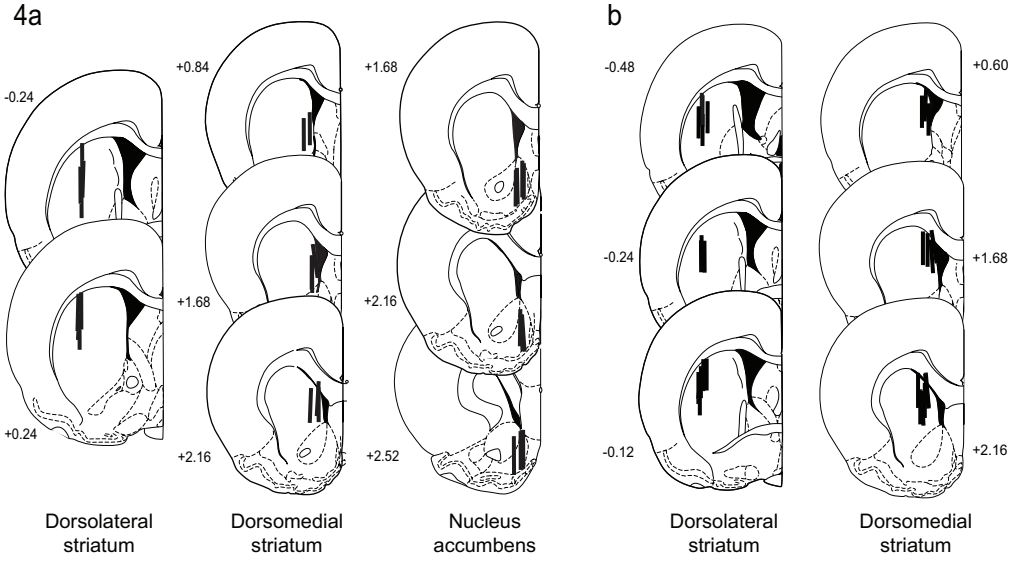


From Experiment 2, the dose-response effect of cumulative infusions of ethanol and saline on dialysate dopamine in (a) the dorsomedial striatum and (b) the dorsolateral striatum, represented as a percent of baseline. Asterisks (*) indicate statistical significance ($p<0.05$) relative to baseline and relative to carets (^) indicate statistical significance ($p<0.05$) between the ethanol and saline groups at specific time points. Symbols represent the mean. Error bars are SEM, but not all are shown for clarity.

Histologies and calcium-dependency

Histological analyses were performed to confirm probe placements, and for inclusion in the final dataset, probes were required to have at least 50% of the active area in the region of interest (Figure 10). Additionally, all animals had at least 50% calcium dependent dopamine release (range: 55-89% calcium dependency).

Figure 10: Histologies to show probe placements



Histologies to show probe placements for (a) experiment 1 and (b) experiment 2. Each line represents the 1.5 mm active area of a single probe.

DISCUSSION

The current study is the first to compare the effects of acute systemic ethanol on dorsomedial and dorsolateral striatal dopamine activity. In experiment 1, we showed that striatal subregions significantly differ in their extracellular dopamine responses to a single dose of intravenous ethanol (1 g/kg), with the nucleus accumbens demonstrating an immediate response to the drug administration that was more robust than either the dorsomedial or dorsolateral striatum. In experiment 2, our findings indicate an overall dose-dependent effect of non-contingent, systemic ethanol on dorsal striatal dopamine, and this stimulation was driven by a stronger effect in the dorsomedial striatum (Figure 3). While we observed slightly higher basal dopamine concentrations in the ethanol groups relative to the saline groups in experiment 2, this difference was not statistically significant. However, in order to directly compare drug effects, we reconciled the differences in basal dopamine by transforming the raw data to percent of baseline (Figure 9).

The present work is the first to demonstrate that the direct pharmacological effect of acute ethanol on extracellular dopamine differs across dorsal striatal subregions. Additionally, the present work supports the previously reported difference between the ventral and dorsal striatum in the dopamine response to acute ethanol (Imperato & Di Chiara, 1986; Melendez et al., 2003). The use of intravenous ethanol administration minimizes the impact of handling stress, motivation, and behavior on striatal dopamine activity. Therefore, the

subregional differences we observed reflect the direct pharmacological effects of ethanol. Here we report that administration of a single dose of ethanol (1 g/kg) produces an immediate and robust increase in extracellular dopamine content to about 140-150% of basal levels in the nucleus accumbens, which is consistent with previous work (Howard et al. 2008; Melendez et al. 2003; Yim & Gonzales 2000b; Imperato & Di Chiara 1986). Furthermore, the temporal profiles of extracellular dopamine in the dorsomedial striatum and dorsolateral striatum starkly contrast that of the nucleus accumbens following a bolus of ethanol. In the nucleus accumbens, a 1 g/kg infusion of ethanol produces an immediate transient increase in dopamine. Conversely, in the dorsomedial striatum we observe a gradual, but non-significant increase in dopaminergic tone, and little or no effect of ethanol in the dorsolateral striatum.

Previous in vivo microdialysis studies in rats have also demonstrated a blunted dopamine response in the dorsal striatum relative to the ventral striatum following acute systemic ethanol administration (Imperato & Di Chiara, 1986; Melendez et al., 2003). Melendez and colleagues (2003) demonstrated that following a single dose of ethanol (2.25 g/kg, i.p.), dopamine peaks at 198% and 156% of baseline levels in the nucleus accumbens and dorsal striatum, respectively. Additionally, Imperato and DiChiara (1986) reported that low doses of ethanol (0.25 and 0.5 g/kg, i.p.), which stimulated accumbal dopamine activity to 135-180% of basal levels, failed to stimulate dopamine activity in the dorsal striatum. Higher doses of ethanol (2.5 and 5.0 g/kg, i.p.) also had a greater effect

on extracellular dopamine content in the nucleus accumbens relative to the dorsal striatum.

The work presented here is consistent with these earlier studies; however, we further delineate the effect of cumulative ethanol doses on dopamine in dorsal striatal subregions. With higher doses of ethanol (in the range of 1.75-2.5 g/kg), we observe enhanced dopaminergic tone in the dorsomedial striatum that stabilizes at 130% of basal dopamine concentrations and endures as brain ethanol levels decline. In contrast, we observed little to no effect of ethanol on dopamine in the dorsolateral striatum at any dose. The overall conclusion from these experiments is that dopamine neurons projecting to the dorsomedial striatum and dorsolateral striatum show differential sensitivity to a systemic ethanol challenge. However, a caveat is that the difference in the response to acute ethanol between these subregions did not reach statistical significance in all of our analyses, suggesting that the overall difference between these two regions is not very robust.

The current data add to the body of literature suggesting that midbrain dopamine circuits differ in their sensitivity to the stimulant effects of ethanol (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985; Melendez et al., 2003; Vena & Gonzales, 2014). Gessa et al. (1985) were the first to report that ethanol stimulated ventral tegmental area dopamine neuron firing rate in vivo with higher potency relative to substantia nigra dopamine neurons in awake, paralyzed rats. These investigators showed that the ethanol dose-response curve was shifted to

the right for electrodes placed in the substantia nigra compared to the ventral tegmental area. Microdialysis studies by Melendez et al. (2003) also provide evidence for a higher ethanol sensitivity of ventral tegmental area dopamine neurons compared with those in the substantia nigra in awake, behaving rats. They showed that an injection of ethanol (2.25 g/kg, i.p.) in naïve rats produced a significant dopamine response in the ventral pallidum, which receives dopaminergic input primarily from the ventral tegmental area. In contrast, no dopamine response to ethanol was observed in the globus pallidus, which receives dopaminergic input primarily from the substantia nigra (H. Fuchs & Hauber, 2004; Lindvall & Björklund, 1979; Prensa & Parent, 2001). The differential sensitivity of ventral tegmental area and substantia nigra dopamine neurons to ethanol likely contributes to the differences we report here in the magnitude and temporal profile of the dopamine response to acute ethanol among the dorsomedial striatum, dorsolateral striatum, and nucleus accumbens. In rats, dopaminergic projections to the dorsomedial striatum and dorsolateral striatum arise primarily from distinct groups of neurons within the substantia nigra, but the dorsomedial striatum also receives some innervation by dopamine neurons with cell bodies located in the ventral tegmental area (Gerfen, Herkenham, & Thibault, 1987; Joel & Weiner, 2000). This ventral tegmental area innervation of the dorsomedial striatum may explain, in part, our finding of a dose-dependent stimulation of dopamine release by ethanol in the dorsomedial striatum, but not in the dorsolateral striatum. Interestingly, differences in the

magnitudes and temporal profiles of the dopamine responses between the dorsomedial and dorsolateral striatum have also been reported following electrically stimulated dopamine activity. Following stimulation of the medial forebrain bundle, the dopamine response in the dorsomedial striatum is larger than that in the dorsolateral striatum (Taylor et al., 2015), which is similar to what we observe following acute ethanol administration.

The mechanisms that underlie the apparent increased sensitivity of the ventral tegmental area dopamine neurons to ethanol relative to the substantia nigra are unknown. Numerous possibilities could contribute to this differential vulnerability to ethanol, and a complete exploration of these possibilities is beyond the scope of the present report. However, the literature provides some clues that would need further experimentation to verify. For example, recent work has demonstrated functional diversity between substantia nigra and ventral tegmental area dopamine neurons in motivated behaviors, which may be due to regulation by contrasting sources of excitatory input (Masayuki Matsumoto & Hikosaka, 2009; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012). Substantia nigra dopamine neurons primarily receive input from somatosensory and motor cortices in addition to the subthalamic nucleus. On the other hand, ventral tegmental area dopamine neurons receive projections from the lateral hypothalamus. These projections from the lateral hypothalamus are rich in neuropeptides, which induce burst firing in ventral tegmental area neurons (Korotkova, Ponomarenko, Brown, & Haas, 2004; Watabe-Uchida et al., 2012). It

is possible that ethanol may be modulating activity at or upstream from these excitatory synapses in the ventral tegmental area while having minimal effects at afferents to the substantia nigra. However, additional distinctions exist between ventral tegmental area and substantia nigra dopamine neurons including receptor expression patterns and mechanisms of terminal regulation in projection regions (Cass & Gerhardt, 1995; Cass et al., 1993; Korotkova et al., 2004; Roeper, 2013). Further work is clearly needed to determine if the differences ethanol sensitivity between the dorsomedial striatum and dorsolateral striatum we observed in the present study could be due to ethanol's direct effects on dopamine cell bodies, afferents regulating midbrain dopamine activity, effects at the level of the dopamine terminals in the striatal subregions, or some combination of these factors.

In conclusion, our microdialysis experiments indicate differential pharmacological effects of acute ethanol on extracellular dopamine in the dorsomedial striatum, dorsolateral striatum, and nucleus accumbens. Specifically, the temporal profiles and magnitudes of the dopamine response to an intravenous bolus of ethanol significantly differed across these regions. Therefore, our results indicate distinctions in the regulation of extracellular neurochemical activity among the dorsomedial striatum, dorsolateral striatum, and nucleus accumbens, which may contribute to their proposed functional distinctions. Furthermore, the doses used in the present studies induced varying degrees of ataxia and sedation, suggesting that they are acutely intoxicating

(Majchrowicz, 1975). Therefore, our findings suggest a role for the dorsomedial striatum in mediating the intoxicating effects of acute ethanol.

Chapter 4:

Medial Prefrontal Cortical Dopamine Responses During Operant Self-Administration of Sweetened Ethanol

The work presented in this chapter was published in 2016 in Alcoholism: Clinical and Experimental Research by Doherty, Schier*, Vena*, Dilly and Gonzales (*denotes equal contributions). This publication is reprinted with permissions from John Wiley & Sons (License #: 3994840195169). Parts of this work have also been published in a dissertation by Dr. Schier. Ashley Vena conducted the independent replication project, edited the manuscript, and prepared the manuscript for final publication.*

ABSTRACT

Medial prefrontal cortex (mPFC) dysfunction is present in heavy alcohol consumers. Dopamine signaling in mPFC is associated with executive functioning and affects drinking behavior; however, direct measurement of extracellular mPFC dopamine during appetitive and consummatory ethanol self-administration behavior has not been reported. We used in vivo microdialysis in freely-behaving, adult, male, Long Evans rats to determine extracellular dopamine concentration in the mPFC during operant self-administration of an ethanol-plus-sucrose or sucrose solution. The model separated appetitive/seeking from consummatory phases of the operant session. Dopamine was also monitored in an untrained handling control group, and

dialysate ethanol was measured in the ethanol-drinking group. Home cage baseline dopamine was lower in rats that experienced a week of drinking sweetened ethanol compared to sucrose-drinking and handling controls. Transfer into the operant chamber and waiting to drink, and cues/initiation of consumption stimulated a relatively higher change in dopamine over baseline in the sweetened ethanol group compared with sucrose and handling controls. However, all groups show a dopamine response during transfer into the operant chamber, and the sucrose group had a relatively higher change in dopamine over baseline during initiation of consumption compared to handling controls. The time courses of dopamine and ethanol in the mPFC differ in the ethanol-consuming rats. Differences in extracellular mPFC dopamine between ethanol drinkers compared to control groups suggest that mPFC dopamine is involved in the mechanism of operant self-administration of sweetened ethanol and sucrose. Furthermore, the increase in dopamine during consumption is consistent with a role of mPFC dopamine in reward prediction.

INTRODUCTION

Medial prefrontal cortex (mPFC) dysfunction, which is frequently noted in heavy alcohol consumers, is associated with increased impulsivity and perseveration, as well as deficits in executive functions (e.g. working memory, decision-making, attention, goal-directed behavior; Bechara & Damasio, 2002;

Bechara & Van Der Linden, 2005; Chanraud et al., 2007; Goldstein et al., 2004; Sullivan et al., 1993). Similarly, acute ethanol can also disrupt working memory (Ralevski et al., 2012). When exposed to drug-related cues, detoxified alcoholics showed significantly greater mPFC activation compared to controls (Heinz et al., 2004). These findings suggest that ethanol use may be associated with enhanced sensitivity to drug-related cues and decreased behavioral control due to compromised prefrontal cortical function.

The mechanisms by which ethanol alters mPFC function are not clear, although recent work has suggested that ethanol-induced changes in dopaminergic activity in the mPFC may contribute (Z.-M. Ding, Ingraham, Rodd, & McBride, 2015; Trantham-Davidson et al., 2014; Tu et al., 2007). Altered dopaminergic signaling has been shown to affect reinstatement of drug-seeking behaviors (Kehagia, Murray, & Robbins, 2010; McFarland, Davidge, Lapish, & Kalivas, 2004; Sinha, 2013), however, the relationship between mPFC dopamine and ethanol self-administration behavior is still unclear. Recent studies show that ethanol increases extracellular dopamine in the mPFC in naïve rats after intravenous administration (Schier et al., 2013), or a single microinjection of ethanol directly into the ventral tegmental area (VTA; Ding et al., 2011), although earlier work suggested that ethanol administration had no effect (Bassareo et al., 1996; Engleman, Ingraham, McBride, Lumeng, & Murphy, 2006; Hegarty & Vogel, 1993). Furthermore, modulation of dopamine receptor signaling in the

mPFC has been shown to change ethanol self-administration behaviors (Ding et al., 2015; Hodge et al., 1996; Samson & Chappell, 2003), but due to the experimental designs used it is unclear if mPFC dopamine was important for drug-seeking or consummatory behaviors.

Previous work indicated that ethanol-associated stimuli can increase extracellular dopamine in the nucleus accumbens (NAC) during operant sweetened ethanol self-administration (Carrillo & Gonzales, 2011; Doyon, Anders, Ramachandra, Czachowski, & Gonzales, 2005; Elaina C Howard, Schier, Wetzel, & Gonzales, 2009a), and therefore, we hypothesized that extracellular dopamine would also increase in the mPFC when an experienced, non-dependent, rat is exposed to drinking-associated stimuli. To test this, we used microdialysis to monitor extracellular dopamine within the mPFC during an operant self-administration session, in which rats drank a 10% sucrose plus 10% ethanol solution (10S10E), a 10% sucrose-only solution (10S; to control for sucrose in the ethanol solution), or no solution (Handling; to control for experimenter handling and experience within the operant chamber). Additionally, our operant self-administration sessions separated anticipatory/seeking and consummatory behavioral phases. Thus, we monitored mPFC dopamine concentrations affected by contextual cues during transfer into the drinking environment (operant chamber) before exposure to stimuli experienced during the drink period (taste, smell, and consumption).

MATERIALS AND METHODS

Materials

Drinking solutions (10S: 10% sucrose (w/v) or 10S10E: 10% ethanol (v/v) in 10S) were made from 95% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY), ultra-pure sucrose (MP Biomedicals, LLC, Solon, OH) and distilled water. Carprofen (Pfizer, New York, NY) and gentamicin (APP Pharmaceuticals, Schaumburg, IL) were used during surgery.

Animals

Final statistical analyses used 27 male, young adult, Long Evans rats from Charles River Laboratories (Portage, MI or Raleigh, NC, USA; 200-225 g upon arrival). An additional 16 male Long Evans rats were used for an independent replication study (see Supplementary Materials). Animals were maintained on a 12-hour light/dark schedule, at 23 ± 2 ºC, with ad libitum food and water (except where noted); rats were weighed each day. All animal procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Surgery

After a week of habituation to the facility and experimenters, rats were anesthetized with isoflurane (5% induction, 2.5% maintenance), and using stereotaxic equipment a 21-gauge guide cannula was surgically placed (Plastics One, Roanoke, VA) above the left mPFC (mm relative to bregma and skull surface: +3.0 AP, +0.6 ML, -2.0 DV (Paxinos & Watson, 2007). Three skull screws and dental cement secured the cannula and a tether bolt to the skull. We administered carprofen (5 mg/kg, subcutaneously) to minimize post-surgical malaise, placed a dummy cannula into the cannula to prevent blockage, and monitored weight and health over a seven-day recovery period prior to beginning operant training.

Self-Administration Training and Protocols

Groups

Rats were initially trained to lever press for access to the 10S solution, and then two groups were formed, one that consumed increasing concentrations of ethanol (2-10% ethanol (v/v) in 10S; Table 3), and a group that continued to drink 10S. The Handling control group was exposed to all the same procedures as the 10S10E and 10S groups (physical handling, water deprivation during lever

press training, time in the operant chamber, tethering, and dialysis), but they were not exposed to drinking solutions or operant training.

Lever-press training and operant protocol

A week after surgery, animals were habituated to operant chambers (Med Associates, Inc., Vermont, USA) and then trained to lever press for a 10S solution (10S10E and 10S groups only). Water deprivation (maximum 22 hours/day) was used to expedite lever-press training. Animals typically learned to lever press within three training sessions (one session/day), after which they regained ad libitum access to water for the remainder of the experiment. Chambers were as previously described by Howard et al. (2009). Briefly, chambers had a retractable lever, sipper tube bottle, house light, cue light and lickometer circuit. Chambers were contained in sound-attenuating boxes. Operant programs were run and data were collected using Med Associates software.

Once trained to lever press, animals began an eight-session testing schedule during which a pre-lever-press wait period was lengthened from 0-28 min, and the response requirement was increased from 2-4 (Table 3). Following completion of the response requirement, the sipper tube containing the drinking solution entered the chamber for 21 minutes, during which animals had ad libitum access to the drinking solution. No further responding was required. For the 10S10E group, ethanol started at a 2% (v/v) concentration in 10% (w/v) sucrose,

and gradually increased to 10% ethanol in 10% sucrose (10S10E) (Table 3). This procedure is modified from the Samson (1986) sucrose fading procedure; however, we did not fade sucrose out of the solution because we wanted to maximize ethanol consumption. Sessions were run once a day, four to six days per week. Animals received a total of three to four, but never more than two sequential days off from training once the eight-session protocol began. The handling control group completed the same procedures, except drinking solutions were not available, and the lever was present but pressing had no consequences. Solution consumption was measured by the volume of solution before and after the drinking session (to the nearest 0.25 ml, accounting for spillage), and pattern of consumption was monitored using the lickometer.

Following the sixth operant session, a spring was attached to the tether bolt on the animal's head and connected to a swivel suspended above the rat by a counter-balance lever arm. Rats were tethered in their home cages (placed next to their operant chamber) and during the seventh operant session to facilitate habituation to the apparatus and environment. The tethering apparatus did not interfere with the rats' abilities to move freely about their home cage or to lever press in the operant chamber.

Microdialysis

After the seventh operant session, rats were briefly anesthetized with isoflurane to implant the lab-constructed microdialysis probe (3.25 mm active area, 13,000 MW cutoff, constructed similar to Pettit and Justice, 1991). Probes were perfused with artificial cerebral spinal fluid (ACSF: 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.2 mM ascorbic acid, and 5.4 mM D-glucose), at a 0.2 µl/min flow rate overnight, and then to a 1.0 µl/min flow rate at least two hours prior to dialysis sampling. For the 10S10E group, two samples before the lever extended into the chamber and all samples after were evaluated for ethanol concentration (described below). The dialysis samples were immediately frozen on dry ice and stored at -80 ºC until dopamine analysis.

Table 3: 8-session protocol parameters

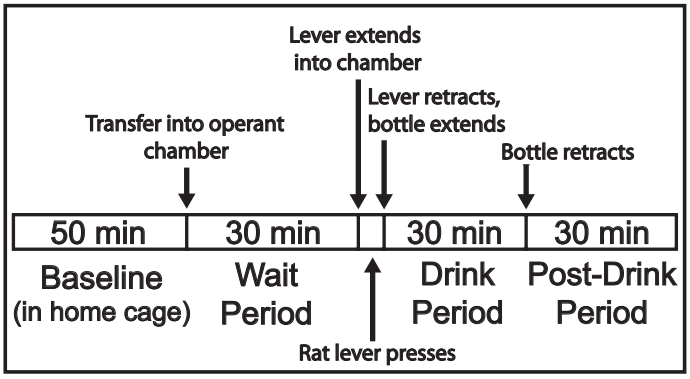
Training Day	Wait Period (min)	Lever Presses Required	Ethanol in 10% Sucrose Drinking Solution	Ethanol Group Intake (g/kg)*	Sucrose Group Intake (g/kg)*
1	2	2	0%	n/a	2.94 ± 0.31
2	5	2	2%	0.43 ± 0.05	2.99 ± 0.30
3	8	2	2%	0.57 ± 0.06	2.88 ± 0.36
4	13	2	5%	1.06 ± 0.10	2.78 ± 0.55
5	18	4	5%	1.07 ± 0.17	3.44 ± 0.31
6	23	4	10%	1.12 ± 0.18	3.63 ± 0.34
7 (Tethered)	28	4	10%	1.28 ± 0.12	3.56 ± 0.28
8 (Dialysis)	28	4	10%	1.65 ± 0.14	3.27 ± 0.38

10S10E refers to the 10% sucrose + 10% ethanol solution the ethanol-consuming rats drank the day of dialysis. Sucrose-consuming rats drank only the 10% sucrose solution (10S), and the handling group did not receive a drinking solution and are not required to lever press. *Data are the mean g/kg ± SEM.

Experimental Timeline

Microdialysis samples were manually collected every seven minutes before and during the eighth operant session (Figure 11). Four baseline samples were taken in the home cage. During the last minute of the fourth baseline sample, the rat was transferred into the operant chamber. The operant program began with turning on the house light and sound-attenuating fan, and the sample collection vial was changed to the first wait period sample. Four wait period samples were taken. The time it took the rat to meet the response requirement of four lever presses was collapsed into the last wait period sample. The wait/lever-press sample was changed to the first drink sample as the drinking bottle entered the chamber. Three samples were taken during the drink period, after which the bottle retracted and the house light turned off. Then three samples were taken during the post-drink period. The rat was then returned to its home cage, and the ACSF was changed to calcium-free ACSF. Approximately 1.5 hours later, two additional calcium-free samples were taken.

Figure 11: Time course of the operant self-administration session



On the eighth day of operant testing, consecutive seven-minute dialysis samples were taken during all behavioral phases. Figure adapted from Schier et al., 2013.

Dopamine Analysis

We evaluated the dopamine concentration in all samples using reverse-phase high performance liquid chromatography with electrochemical detection. All samples were run with accompanying external standards (0.03125 to 1.0 nM dopamine). Samples and standards were run using a 8125 manual injector (Rheodyne, Cotati, CA), a Luna 50 x 1.0 mm (C18, 3-µm particle size; Phenomenex, Torrance CA) or Haisil 100 50 x 0.5 mm column (C18, 3-µm particle size; Higgins Analytical Inc., Mountain View, CA), and a 2 mm glassy carbon working electrode (SenCell or VT03 with ISAAC reference electrode, Antec Leyden, Netherlands) at potential + 345 or + 395 mV. Mobile phase aqueous solution consisted of approximately 2.1 mM octanesulfonic acid, 0.04 - 0.3 mM decanesulfonic acid (adjusted to optimize chromatography), 0.34 mM ethylenedianimetetraacetic acid, 71 mM sodium phosphate monobasic dihydrate,

and 60 mM potassium chloride, adjusted to 5.60 pH with 1 M sodium hydroxide. Prior to use, 150 mL/L of methanol was added and the mobile phase solution was sparged with helium. Mobile phase flow rates ranged from 0.1 to 0.12 ml/min for 50 x 1.0 mm columns, and 0.025 – 0.032 ml/min for 50 x 0.5 mm columns. Four to 5.5 µl of dialysate was mixed with 1 to 3 µl of ascorbate oxidase (EC 1.10.3.3; 102.3 U/mg) prior to injecting 5 µl of the mixture into the system. The amount of ascorbate oxidase was adjusted to optimize dopamine detection. EZChrome Elite software (Agilent Technologies, Wilmington, DE) was used for chromatogram acquisition and peak integration. The dopamine signal was required to be at least 3 times greater than the background noise. See Supplementary Materials for details regarding the dopamine analyses for the independent replication study.

Ethanol Analysis

Samples were analyzed for ethanol concentration on the day they were collected (Schier, Mangieri, Dilly, & Gonzales, 2012). Briefly, 1 µl aliquots of dialysate or external standards (0.3125 to 20 mM ethanol) were sealed in 2 ml glass vials, heated in an autosampler tray (50-65 ºC), and analyzed for ethanol content by a gas chromatograph with flame ionization detection.

Histological Analysis

Within three days of dialysis, animals were overdosed with sodium pentobarbital (150 mg/kg, intraperitoneal) and perfused through the heart with saline and 10% formalin in saline prior to brain extraction. Brains were post-fixed with 10% formalin in saline, coronally sectioned (100 μm thick), and stained with cresyl violet for verification of the microdialysis probe placement (Paxinos et al., 1999).

Exclusion Criteria

For inclusion of rats in data analysis, dopamine concentrations in home cage baseline samples were required to have a relative standard deviation < 0.25. We also required a 40% decrease in dopamine concentration in calcium-free ACSF samples compared to basal ACSF samples to verify that dopamine release was exocytotic. Rats were required to acquire the lever-press behavior within six training sessions, complete the lever press requirement on the day of dialysis, and the 10S10E group was required to consume at least 0.8 g/kg on the day of dialysis. Finally, rats were excluded if technical errors resulted in loss of critical dopamine samples (before and after transfer into the operant chamber, or initiation of drinking).

Statistical Analysis

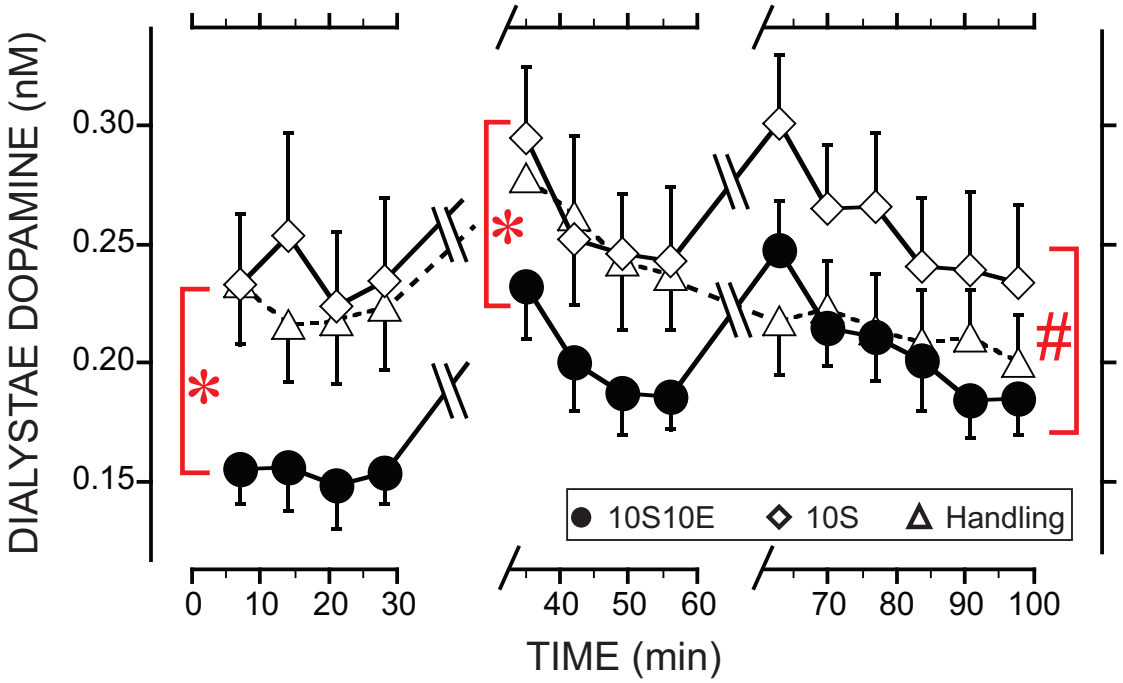
Raw dopamine concentrations (nM) were analyzed using repeated measures analysis of variance (ANOVA) with group (10S10E, 10S, and handling) as between subjects factor, and time (14 time points: 4 home cage baseline, 4 wait period, 6 drink and post-drink) as within subjects repeated factor. Post hoc analyses (using pooled error and Bonferroni corrections) separated the experiment into three phases: home cage baseline, wait period, and drink/post-drink. Specifically, when significant interactions between main effects were observed for the overall experiment, simple effects analyses were done to determine the source of the significant interaction. Since significant group differences occurred in raw dopamine concentrations during the home cage baseline (see results; Figure 2), we also analyzed dopamine expressed as a percentage of home cage baseline levels. Behavioral data were analyzed using independent samples t-tests; except licks separated into seven minute bins were analyzed with repeated measures ANOVA with group (10S10E and 10S) as between subjects factor, and bin as within subjects repeated factor. Data were analyzed using SPSS software (IBM). Significance was assigned if $p < 0.05$; ns= not significant.

RESULTS

Consumption during operant self-administration sessions

Ethanol and sucrose consumption data for the eight operant sessions are represented in Table 3. In the group consuming sweetened ethanol, rats increased their ethanol intake over the eight sessions and consumed at least 1 g/kg during the four sessions prior to microdialysis.

Figure 12: Dialysate dopamine concentrations in mPFC during home cage baseline, wait, drink and post-drink periods for the sucrose and handling controls along with rats trained to drink 10S10E.



For clarity only group comparisons are shown on the figure as follows. * indicates a significant difference between the 10S10E group compared with either the 10S or Handling groups during the baseline and wait periods. # indicates that drink and post-drink period dopamine concentrations were significantly different in 10S compared with either the 10S10E or Handling groups. Not indicated in the figure are significant increases in dopamine at the first sample during the wait period compared with the remaining samples. Similarly, there was a significant increase in dopamine during the first drink sample compared with the remaining drink and post-drink samples. Overall significant main effects of time and a group by time interaction occurred. Data represented as mean ± SEM for most points, but selected error bars are omitted for clarity (n=9 for each group).

Overall analysis of raw dopamine concentrations and home cage baseline

In general, dopamine concentrations in both the 10S10E and 10S groups peaked once during the first wait period time point, and again during the first drink period time point. In contrast, dopamine in the Handling group only peaked during the first wait period time point and remained at baseline levels during the drink and post-drink periods. Comparison of overall raw dopamine concentrations across the entire experiment (Figure 12) resulted in significant main effects of time ($F_{13,306}$=11.9, $p<0.001$), and a group by time interaction ($F_{26,306}$=3.0, $p<0.001$). Post hoc analyses of the significant group by time interaction separated the experiment into the three phases: home cage baseline, wait period, and drink/post-drink.

Significant group differences occurred in raw dopamine concentrations during the home cage baseline (Figure 12; $F_{2,33}$=12.1, $p<0.001$). Following-up on the main effect of group, an ANOVA revealed that the 10S10E group exhibited significantly lower baseline dopamine concentrations compared to both the 10S ($F_{1,33}$=20.9, $p<0.05$) and Handling groups ($F_{1,32}$=14.8, $p<0.05$), while no difference occurred between 10S and Handling groups. A separate experiment utilizing the same experimental protocol also found that 10S10E animals show significantly lower baseline dopamine concentrations in the PFC compared to 10S controls (t_{14}= 1.76, $p<0.05$; Fig S1)

Raw dopamine concentrations and lever-press behavior during the wait period

After collecting the home cage baseline samples, the rats were transferred into the operant chamber and the program was started. During the wait period (Figure 12), dopamine concentrations significantly differed between groups ($F_{2,33}$=6.3, p<0.01) and changed over time ($F_{3,306}$=12.5, p<0.001), but there was not a group by time interaction (ns). Post hoc analyses on the main effect of group during the wait period showed that dopamine in the 10S10E group significantly differed from both the 10S ($F_{1,33}$=10.1, p<0.05) and Handling groups ($F_{1,33}$=8.6, p<0.05). Dopamine concentrations were similar between 10S and Handling groups during the wait period. Analysis of the change in dopamine during the wait period collapsed across all groups showed that dopamine concentrations peaked during the first wait period time point, and then decreased back towards baseline levels (first sample differed from samples 2-4, p<0.05).

At the end of the wait period, the lever was presented to all groups. 10S10E and 10S groups were required to press the lever four times for access to the drinking solution. The 10S10E group began lever pressing significantly sooner than the 10S group after lever presentation (t_{16}= -2.3, p<0.05), yet both groups showed similar lever-press rates (t_{16}= 1.1, ns), and similar time to complete the lever press requirement (t_{16}= -0.1, ns) (Table 4). The time taken to complete the lever presses was accounted for in the final wait period dialysis sample, making that sampling period variable (7 min 3 sec - 10 min 4 sec).

Table 4: Behavioral parameters

Parameter	10S10E Group	10S Group
Time to complete presses (sec)	24 ± 16	25 ± 3.2
Latency to press (sec)	7.0 ± 3.8*	18.3 ± 3.2
Latency to drink (sec)	6.1 ± 2.2	2.8 ± 0.2
Length of first bout (min)	4.9 ± 0.3*	8.4 ± 1.1
Licks in first bout	1239 ± 80*	2478 ± 310
First bout lick rate (licks/min)	259 ± 21	305 ± 20
Solution consumed (ml)	8.8 ± 0.7*	13.8 ± 0.9
Total licks	1503 ± 100*	2704 ± 265

***** represents significant difference in behavioral parameter between ethanol plus sucrose solution-consuming (10S10E) and sucrose solution-consuming (10S) groups. Data represented as mean ± SEM.

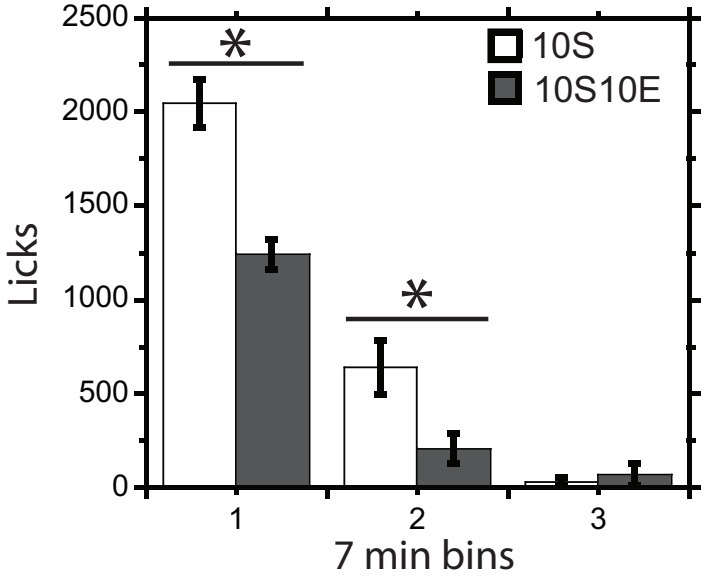
Drinking behavior and raw dopamine concentrations during the drink and post-drink periods

Once the response requirement was completed, the lever retracted and the bottle entered the chamber, at which time the dialysis sample was changed and the drink period began. The 10S10E and 10S groups showed similar latency to drink, rate of licking during the first drinking bout, and total number of drinking bouts (Table 4; bout defined as a minimum of 25 licks without a two-minute break; t_{16}= 1.5, -1.6, -0.7, respectively, ns). The 10S10E group drank significantly less solution, had significantly fewer licks overall and during the first

bout, and had a significantly shorter first bout compared to the 10S group (Table 4; t_{16}= -4.7, -4.2, -3.9, -3.1, respectively, $p < 0.01$). When licks were binned per seven minutes, the 10S10E group had significantly fewer licks during the first two bins compared to the 10S group, but both groups had similar licks during the third bin (Figure 13; group by time interaction $F_{2,32}$=12.4, $p < 0.001$; bin 1: $F_{1,32}$=45.4, $p<0.05$; bin 2: $F_{1,32}$=13.0, $p<0.01$; bin 3: $F_{1,32}$=0.1, ns). On dialysis day, the 10S10E group consumed 1.7 ± 0.1 g/kg ethanol during the drink period (Table 3).

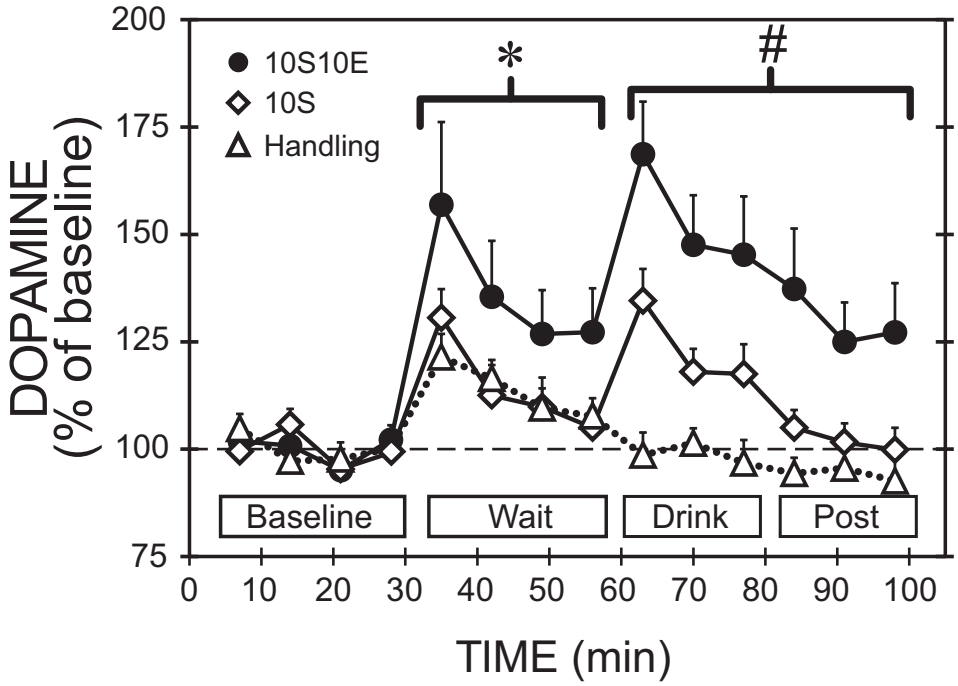
During the drink and post-drink periods (Figure 12), dopamine concentrations significantly differed between groups ($F_{2,33}$=6.9, $p<0.01$) and changed over time ($F_{5,306}$=9.4, $p<0.001$). Following-up on the main effect of group, a post hoc ANOVA revealed that dopamine in the 10S10E group significantly differed from the 10S group ($F_{1,33}$=11.4, $p<0.05$), but not the Handling group (ns). Dopamine concentrations were also significantly different between 10S and Handling groups ($F_{1,33}$=9.2, $p<0.05$). Because there was not a significant group by time interaction in the overall ANOVA conducted on the drink and post-drink periods, the groups were collapsed to analyze the change in dopamine over time during the experimental periods. These analyses revealed that dopamine concentrations peaked during the first drink period time point, and then decreased back towards baseline levels (first drink sample differed from drink sample 3 and post-drink samples 1-3, $p<0.05$).

Figure 13: Licks in seven-minute bins.



Asterisks (*) represent significant difference in licks between 10S10E (n = 9; 10% sucrose + 10% ethanol) and 10S (n = 9; 10% sucrose) groups. Data represented as mean ± SEM.

Figure 14: mPFC dopamine relative to home cage baseline during home cage baseline, wait, drink and post-drink periods.



The data shown in fig. 2 were transformed to percent of home cage baseline. For clarity only group comparisons are shown on the figure as follows. * indicates a significant difference between the 10S10E group compared with either the 10S or Handling groups during the wait period. # indicates that drink and post-drink period dopamine responses above baseline were significantly different in each group compared with the other two groups. Overall significant main effects of time, group, and a group by time interaction occurred. Data represented as mean ± SEM for most points, but selected error bars are omitted for clarity (n=9 for each group).

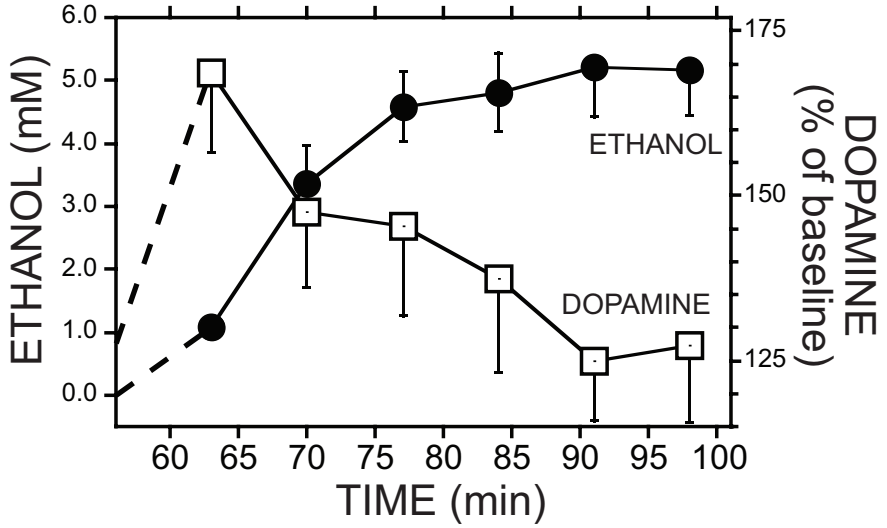
Dopamine concentration as a percentage of home cage baseline levels

Due to significant group differences in raw dopamine concentrations during the home cage baseline (results above; Figure 12), we also analyzed dopamine expressed as a percentage of home cage baseline levels (% BL; Figure 14). In general, analysis of raw and % BL dopamine resulted in similar conclusions, therefore with % BL analysis we mainly describe differences compared to that obtained with raw dopamine concentrations. For example, analysis of all ten time points within the operant chamber (wait period, drink, and post-drink) revealed a significant main effect of group ($F_{2,24}$=7.7, $p<0.01$) in addition to significant time and group by time effects seen with the raw concentration analysis. Simple effects analyses between groups showed that each group significantly differs from one another (10S10E vs. 10S $F_{1,63}$=44.7, $p<0.001$, 10S10E vs. Handling $F_{1,63}$=85.0, $p<0.001$, 10S vs. Handling $F_{1,63}$=6.4, $p<0.05$).

Following up on these simple effects analyses, we identified where significant group differences occurred within each experimental phase. During the wait period, the % BL analysis revealed similar results compared to raw dopamine concentrations. During the drink and post-drink periods, the % BL analysis also revealed similar results compared to raw dopamine concentrations, with significant main effects of group and time. However, an ANOVA on the %BL data also revealed a significant main effect of group, with significant differences

between 10S10E and Handling groups ($F_{1,30}$=81.2, p<0.05), and a significant group by time interaction ($F_{10,115}$=3.0, p<0.01). Subsequent simple effects analysis of the group by time interaction showed that dopamine concentrations in the 10S10E group differed from the 10S group during the first drink time point ($F_{1,30}$=7.7, p<0.05), and differed from the Handling group during all three drink and the first post-drink time points (p<0.05). Dopamine concentrations in the 10S group only differed from the Handling group during the first drink time point ($F_{1,30}$=8.5, p<0.05).

Figure 15: Dopamine and ethanol concentrations in mPFC during the drink and post-drink periods in the 10S10E group.



Left y-axis shows the dialysate ethanol concentrations (circles). Right y-axis shows the percent change in mPFC dopamine concentration during the drink and post-drink periods relative to home cage baseline (squares, same data shown in Fig. 3). The bottle retracted from the chamber after the third sample. Data represented as mean ± SEM (n=9).

Dialysate ethanol concentrations during and after the drink period

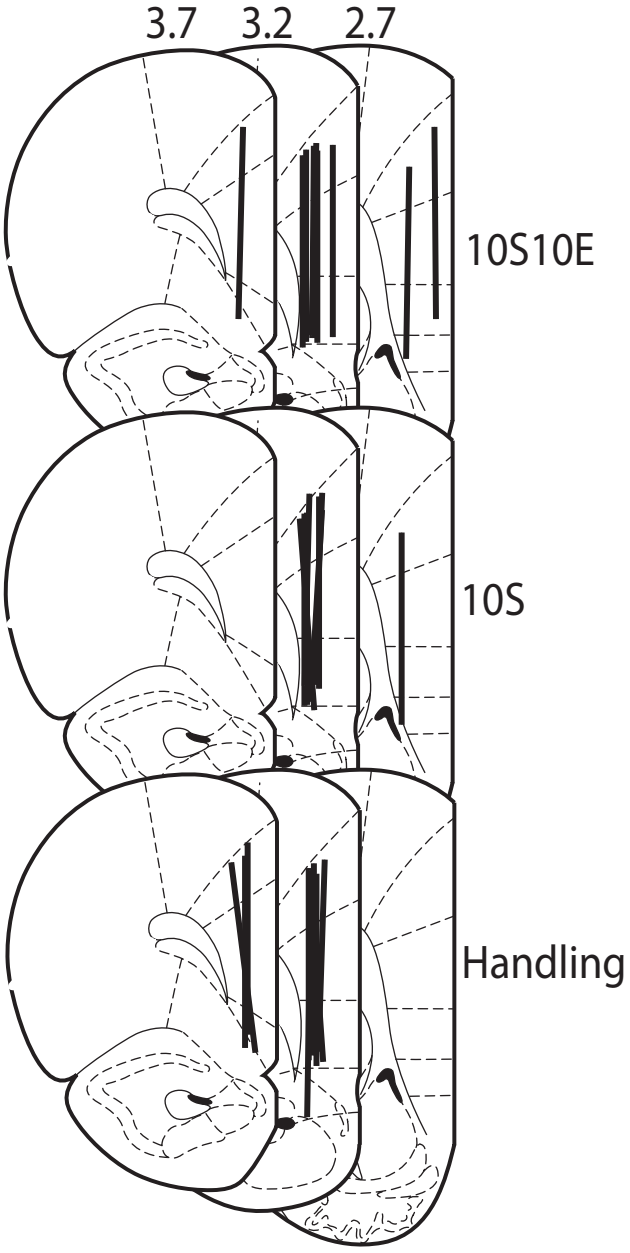
At the initiation of drinking, dialysate ethanol was lowest and then increased throughout the drink and post-drink period to peak at 5.2 ± 0.8 mM ethanol (Figure 15). Dialysate ethanol concentrations are not corrected with an in vivo extraction fraction, and are therefore lower than true tissue ethanol concentrations.

Histology, body weight, and calcium-dependent dopamine concentration

Histologies showed that the percent of probe active area within the infralimbic and prelimbic regions was not significantly different between groups (Figure 16; $F_{2,24}$=2.1, ns). Probe active area was required to be at least 50% in the infralimbic and prelimbic regions of the mPFC. 10S10E, 10S, and Handling groups were 76 ± 3%, 66 ± 5%, and 65 ± 5% within these regions, respectively. Body weights on the day of dialysis were not significantly different between groups ($F_{2,24}$=2.7, ns; range 337-477 grams). Calcium-dependent dopamine release was confirmed by a minimum 40% dialysate dopamine concentration decrease when calcium-free ACSF was perfused through the probe. 10S10E, 10S, and Handling groups showed an average of 67 ± 3%, 57 ± 4%, and 61 ±

5% decrease in dopamine in calcium-free ACSF samples compared to concentrations at the conclusion of the operant session, respectively.

Figure 16: Microdialysis probe placements within the medial prefrontal cortex.



Coronal slices 2.7, 3.2, and 3.7 mm from bregma showing microdialysis probe placements for all experimental groups. Lines represent 3.25 mm active dialysis area. 10S10E = 10% sucrose + 10% ethanol, 10S = 10% sucrose. Histology figure adapted from Paxinos & Watson (1998).

DISCUSSION

This is the first report of changes in mPFC extracellular dopamine during operant self-administration of sweetened ethanol. Dopamine concentrations during the home cage baseline were lower in the mPFC of rats that had experienced about a week of drinking sweetened ethanol vs. sucrose-drinking and handling controls. Upon transfer into the operant chamber, dopamine increased to a greater degree relative to baseline in the mPFC of the sweetened ethanol group compared with sucrose and handling controls; however, all groups showed a dopamine response during the transfer. At the start of the drink period, extracellular dopamine increased in the PFC in both the 10S10E and 10S groups, the magnitude of this effect relative to baseline was greater in the 10S10E group. Overall, we used a behaviorally relevant operant model and show differences in mPFC dopamine concentrations that are unique to rats drinking sweetened ethanol.

Compared to animals in the control groups, rats that had self-administered a sweetened ethanol solution for about a week demonstrated significantly lower basal dopamine concentrations in the mPFC. The reliability of this effect is demonstrated by our own independent replication (Supplementary materials). No baseline mPFC dopamine differences occurred between sucrose-experienced and handling control groups, which highlights the specificity of ethanol experience on mPFC baseline dopamine levels. We speculate that this

limited voluntary ethanol drinking experience may be sufficient to induce synaptic adaptions that alter the regulation of basal extracellular dopamine concentrations in the mPFC, and raises the possibility that lower mPFC dopamine concentration contributes to ethanol-related seeking or drinking behaviors. In the present studies, rats consumed on average 1-1.3 g/kg in the four sessions prior to the microdialysis session. At these doses, it is unlikely that basal dopamine levels were influenced by the aversive, lingering effects of alcohol intoxication during the microdialysis sessions. Previous work has demonstrated that with high doses of acute ethanol (3 or 4 g/kg), rats show conditioned place aversion 10 hours post-ethanol administration, but this behavior is not observed with lower doses (2 g/kg; Morse, Schulteis, Holloway, & Koob, 2000). Furthermore, we did not observe a relationship between ethanol intake on the day prior to microdialysis and basal mPFC dopamine concentrations (data not shown). This supports our argument that the observed reduction in basal mPFC dopamine concentrations in the ethanol-experienced animals is related to repeated self-administration of intoxicating doses of ethanol and not to the lingering effects of the dose consumed the day prior to microdialysis.

Consistent with the suggestion of ethanol-specific effects on basal PFC dopamine, is the report of lower basal dopamine concentrations in mPFC of naïve alcohol-preferring “P” rats compared to the outbred Wistar strain (Engleman et al., 2006). However, not all alcohol-preferring strains of rats

demonstrate reduced basal dopamine activity in the mPFC relative to controls. For example, Leggio et al. (2003) demonstrated higher basal dopamine content in the mPFC of Sardinian alcohol-preferring (sP) rats compared to Wistar control rats. Furthermore, we did not observe baseline dopamine differences in the nucleus accumbens (NAC) between ethanol-experienced rats and controls using similar procedures (Doyon et al., 2005; Howard et al., 2009). This clear contrast between mPFC and NAC suggests that low tonic basal dopamine concentrations selectively in the mPFC might be important for operant self-administration of ethanol.

Differences between ethanol and sucrose drinking groups, and handling controls were noted in the dopamine response during transfer from the home cage into the operant chamber. Rats in all three groups exhibited peak increases in mPFC dopamine when transferred into the operant chamber, and the largest increase in dopamine relative to baseline occurred in the 10S10E group. In contrast, we did not observe a difference between sucrose and handling groups. Across all groups, we attribute some of the dopamine increase to the physical handling of the rat and environment change. Physical handling increases extracellular dopamine in the mPFC, as does transfer into a novel environment (Feenstra, Botterblom, & Mastenbroek, 2000; Feenstra & Botterblom, 1996; Feenstra, Botterblom, & van Uum, 1998). While the operant chamber environment was not novel, it was an environment change. In both ethanol and

sucrose groups, transfer into the operant chamber exposed the rats to reward-associated contextual cues that could have contributed to the observed stimulation of mPFC dopamine. The present data along with previous studies of the NAC (Doyon et al., 2005; Howard et al., 2009), suggest that both regions respond to ethanol-associated contextual stimuli with significant increases in extracellular dopamine relative to baseline compared to sucrose controls. Therefore, dopamine may be acting in the mPFC and NAC to stimulate ethanol-seeking behavior in response to ethanol-associated stimuli.

Our data clearly show that dopamine activity is enhanced in the mPFC during operant self-administration of sweetened ethanol or sucrose alone. Since both solutions are rewarding and can act as reinforcers, the current work provides novel data to support the reward prediction role of mPFC dopamine, as has been found for the NAC (Carrillo & Gonzales, 2011; Day, Roitman, Wightman, & Carelli, 2007; Doyon et al., 2003b, 2005; Doyon, Howard, Shippenberg, & Gonzales, 2006; Howard, Schier, Wetzel, & Gonzales, 2009b; Stuber et al., 2008). The original reward prediction theory arose, in part, from experimental findings of single unit recording of presumed dopamine cell activity in the midbrain (Schultz, 1997). However, single unit recording in the midbrain does not allow conclusions about where dopamine release occurs in response to cues that predict reward. Recent work measuring changes in dopamine release in the NAC has added strong support for the role of dopamine as a reward prediction

signal (Brown, McCutcheon, Cone, Ragozzino, & Roitman, 2011; Day et al., 2007; Hart, Clark, & Phillips, 2015; Stuber et al., 2008; Wassum, Ostlund, & Maidment, 2012). The current work using microdialysis of dopamine in the mPFC is also consistent with the idea that dopamine release may also act as a reward prediction signal similar to that in the NAC, at least in a qualitative manner. This was possible through the use of microdialysis followed by chromatographic separation of dopamine from other biogenic amines found in the mPFC. The time course of the dopamine signals in our microdialysis work is much longer (7 min) than observed with single unit recording or fast scan cyclic voltammetry methods in which the reward signals are observed within 10 seconds

We present our dopamine microdialysis data in two forms: raw dialysate concentration and percent of home cage baseline. In general, the results and interpretations are similar for both analyses across the different phases of the experiment. However, there are minor, but critical differences that should be noted. Specifically, the net increase in concentration of dopamine is similar in the ethanol and sucrose groups during the initiation of drinking when analyzed as raw concentration data, but the ethanol group shows a significantly greater increase as a percent of baseline. This is due to lower baseline concentrations in the ethanol group. The biological significance of either way of looking at this dopamine response is not clear, and both could be important. It has been

suggested that dopamine signaling in the mPFC has an optimal level and increases or decreases from this level contribute to cognitive deficits (Cools & D'Esposito, 2011; Floresco & Magyar, 2006). However, without assessing the functional consequences of reduced basal dopamine concentrations in the mPFC of our animals, we cannot make assumptions about the implications of our findings on cognition.

In conclusion, we present novel data using a behaviorally relevant model that within the mPFC the amount of extracellular dopamine is significantly different in rats that drink sweetened ethanol, compared to sucrose and handling controls. Rats trained to drink sweetened ethanol not only demonstrated reduced basal mPFC dopamine concentrations, but also exhibited a different dopamine response in anticipation of the drinking event and once consumption was initiated. Compared to previous reports on the NAC, our results also highlight important regional differences in the dopamine response to ethanol or ethanol-associated cues that are specific to the mPFC. Thus, our current data provides a critical foundation for future studies that may help identify mechanisms behind compromised prefrontal cortical executive function that are frequently observed in heavy alcohol consumers.

SUPPLEMENTARY MATERIALS

This section details the procedures and results of an independent study to assess the reliability of the observation of reduced basal PFC dopamine in ethanol experienced rats relative to control rats. The overall procedures were similar to those described in the manuscript so only key differences are highlighted below.

Materials and Methods

Materials

Drinking solutions were prepared as described in the manuscript, with the exception that tap water was used instead of deionized water.

Animals

Sixteen male, young adult Long Evans rats from Charles River Laboratories (Raleigh, NC, USA; 220-240 g upon arrival). All animal procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin. Stereotaxic surgeries were performed as described in the manuscript to implant a cannula directly above the mPFC (+3.0 AP; +0.5 ML; -1.1 DV).

Self-Administration Training and Protocols

For the replication project, operant self-administration training was conducted as described in the manuscript. However, a handling group was not necessary for this project and therefore animals were assigned to either the ethanol (10S10E) or sucrose group (10S).

Microdialysis

The microdialysis experiment followed the same procedures as described in the manuscript, with a few exceptions discussed below. During the final microdialysis session, only basal samples were collected.

Following the seventh operant session, rats were briefly anesthetized with isoflurane and a lab-constructed microdialysis probe (Pettit and Justice, 1991) was implanted through the guide cannula and into the mPFC. As described above, probes were perfused with ACSF overnight at a flow rate of 0.2 µl/min. Immediately following probe implantation and for the duration of microdialysis sampling, animals remained in their home cage next to their respective operant chamber. The next morning, the flow rate was increased to 1.0 µl/min at least 2 hours prior to the start of microdialysis.

Microdialysis samples were manually collected every six minutes and were immediately frozen on dry ice. After a minimum of 4 samples was collected, the ACSF was changed to calcium-free ACSF. Calcium-free ACSF was perfused through the probe for at least 2 hours and three additional calcium-free samples were collected.

Dopamine Analysis

Dopamine content in each sample was quantified via reverse-phase high performance liquid chromatography (HPLC) with electrochemical detection. All samples were run with accompanying external standards (0.015 to 1.25 nM dopamine). Samples and standards were run using an 8125 manual injector (Rheodyne, Cotati, CA), a Luna 50 x 1.0 mm (C18, 3-µm particle size; Phenomenex, Torrance CA), and a 2 mm glassy carbon working electrode (SenCell, Antec Leyden, Netherlands) at potential + 450 mV relative to a Ag/AgCl reference electrode. Mobile phase was pumped through the HPLC system at a flow rate of 0.2 mL/min using an ISCO 65D syringe pump. The mobile phase consisted of 0.500 g octanesulfonic acid, 0.050 g decanesulfonic acid, 0.128 g ethylenediaminetetraacetic acid, and 11.08 g NaH_2PO_4 dissolved in 1 liter of deionized water, adjusted to 5.60 pH with 1 M sodium hydroxide. Methanol (6-8% v/v) was added to the mobile phase solution as the organic solvent and the solution was sparged with helium. EZChrome Elite software (Agilent Technologies, Wilmington, DE) was used for chromatogram acquisition and peak integration. For three animals, the chromatography software was unable to determine peak heights of the dopamine signal for one or more of the samples. Therefore, peak area was used to determine the dopamine concentrations of all samples collected for these animals. The dopamine signal was required to be at least 3 times greater than the background noise.

Histological Analysis

Within three days of dialysis, animals were overdosed with sodium pentobarbital (150 mg/kg, intraperitoneal) prior to brain extraction. Brains were post-fixed with 10% formalin in saline, coronally sectioned (120 μm thick), and stained with cresyl violet for verification of the microdialysis probe placement (Paxinos et al., 1999).

Exclusion Criteria

For inclusion of rats in data analysis, dopamine concentrations in home cage baseline samples were required to have a relative standard deviation < 0.25. We also required a 40% decrease in dopamine concentration in calcium-free ACSF samples compared to basal ACSF samples to verify that dopamine release was exocytotic. Rats were required to acquire the lever-press behavior within two training sessions and the 10S10E group was required to consume at least 0.24 g/kg in session 5, 0.43 in session 6, and 0.58 g/kg in session 7. These minimum values for consumption were determined from the ethanol consumption data for the animals included in the original experiment.

Statistical Analysis

Raw dopamine concentrations (nM) were analyzed via a one-sided student's t-test to determine differences in basal PFC dopamine content between

the 10S10E and 10S groups. Data were analyzed using SPSS software (IBM). Significance was assigned if $p < 0.05$; ns= not significant.

Results

Consumption during operant self-administration sessions

Ethanol and sucrose consumption data for the seven operant sessions are represented in Table 5. In the group consuming sweetened ethanol, rats increased their ethanol intake over the eight sessions and consumed at least 1 g/kg during the four sessions prior to microdialysis. The consumption data for the 10S10E and 10S groups resembled those of the original experiment.

Table 5: Ethanol and sucrose consumption per session

Training Day	Ethanol in 10% Sucrose Drinking Solution	Ethanol Intake (g/kg)*	Sucrose Intake (g/kg)
1	0%	n/a	2.80 ± 0.65
2	2%	0.27 ± 0.06	2.52 ± 0.31
3	2%	0.47 ± 0.06	3.03 ± 0.43
4	5%	1.06 ± 0.12	3.17 ± 0.45
5	5%	1.05 ± 0.18	2.89 ± 0.47
6	10%	1.43 ± 0.33	3.13 ± 0.60
7 (Tethered)	10%	1.18 ± 0.25	3.00 ± 0.50

* indicates mean ± SEM.

Analysis of raw basal PFC dopamine concentrations

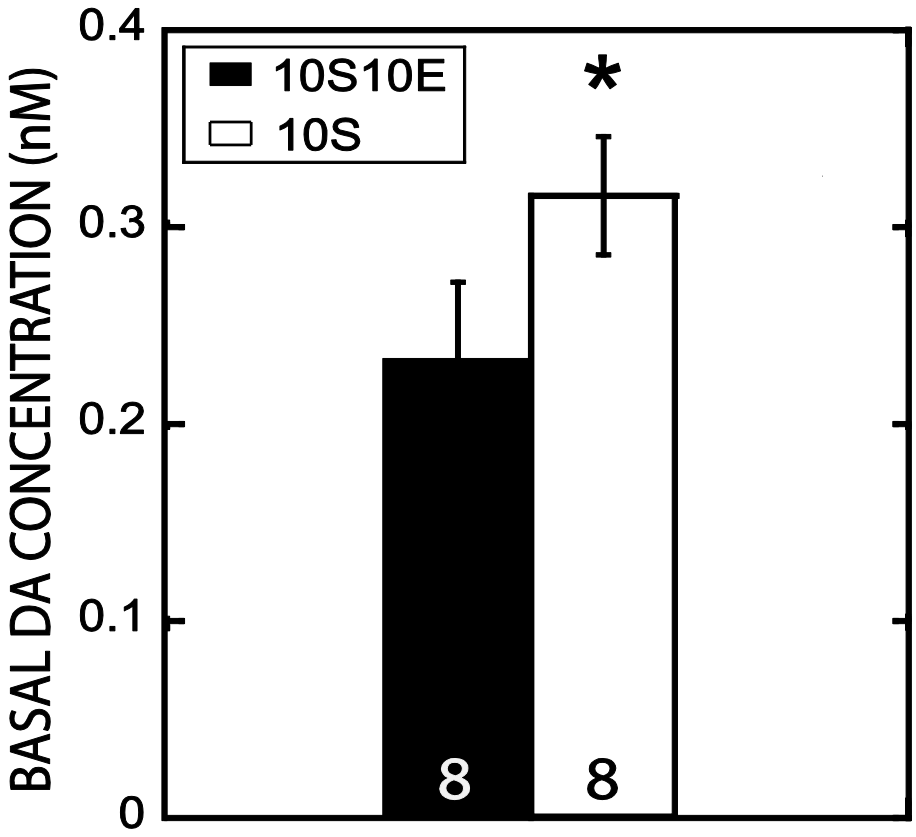
As observed in the original experiment, animals with about a week of sweetened ethanol experience demonstrated significantly lower basal dopamine

concentrations in the PFC relative to sucrose-only controls (t_{14}= 1.76, $p<0.05$; Fig 17).

Histologies and calcium-dependent dopamine concentration

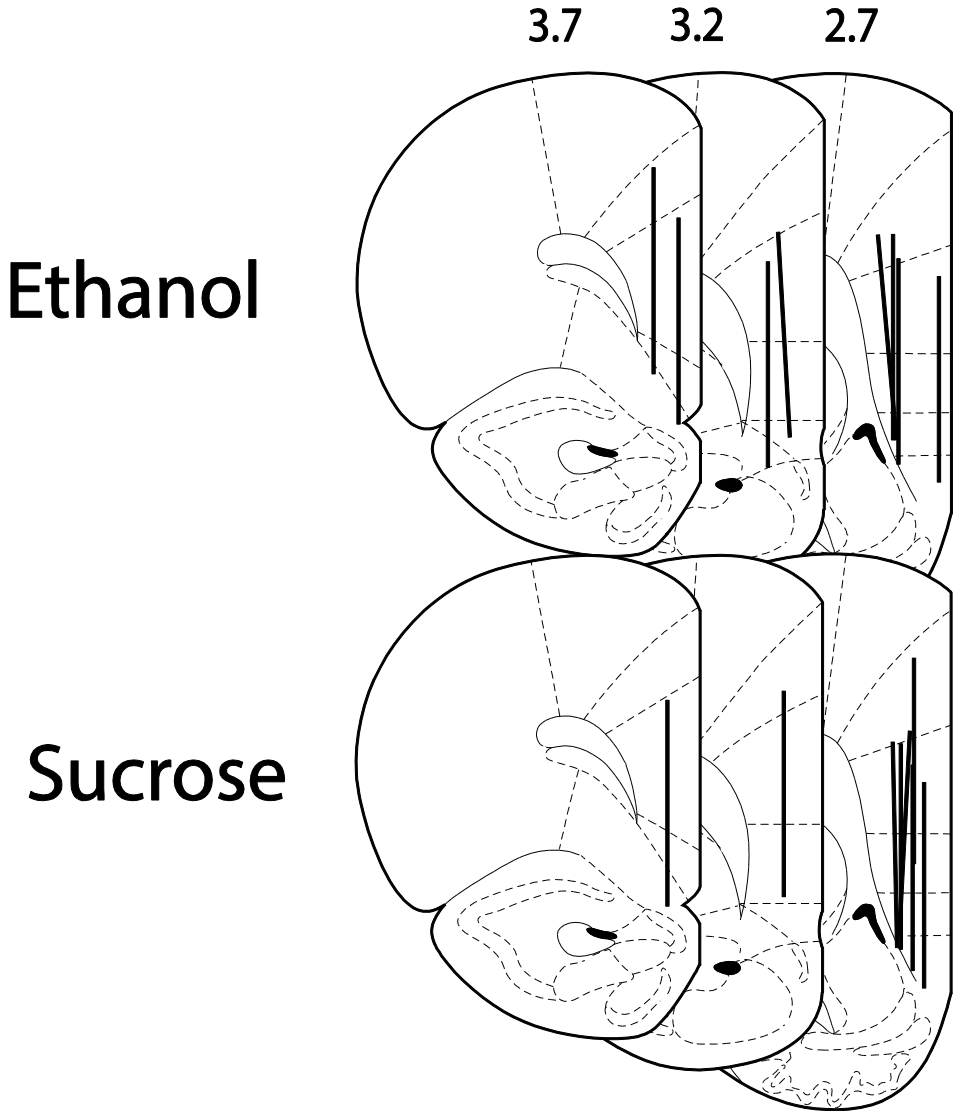
Histologies indicated that at least 50% of the probe active area for each animal was in the infralimbic and prelimbic regions (Figure 18). Calcium-dependent dopamine release was confirmed by a minimum 40% dialysate dopamine concentration decrease when calcium-free ACSF was perfused through the probe. 10S10E and 10S groups showed an average of 63 ± 5% and 57 ± 4% decrease in dopamine in calcium-free ACSF samples compared to concentrations at the conclusion of the operant session, respectively.

Figure 17. Basal dopamine concentrations in the medial PFC of rats trained to consume 10S or 10S10E.



Animals with prior ethanol self-administration experience (10S10E) demonstrate significantly lower basal dopamine concentrations than control rats with only 10S experience. Means ± SEM are represented. *T(14)=1.76, p(10S10E<10S) < 0.05.

Figure 18: Microdialysis probe placements within the medial prefrontal cortex.



Coronal slices 2.7, 3.2, and 3.7 mm from bregma showing placements of the active area (3.0 mm) of the microdialysis probes for each experimental group. Ethanol indicates the group that consumed 10% sucrose + 10% ethanol. Sucrose indicates the group that consumed sucrose only. It should be noted that for one animal in the 10S group, the brain extraction occurred about 72 hours following probe removal. To ensure that a probe track would be visible, the probe was reinserted immediately prior to euthanasia. The animal ended up with 2 separate probe tracks, but based on the extent of the tissue damage, we made an assumption as to which one was the original tract. Histology figure adapted from Paxinos & Watson

Chapter 5:

Quantification of extracellular noradrenergic activity in the mPFC in rats following acute intravenous administration or self-administration of ethanol

The work presented in this chapter has not been previously published.

ABSTRACT

In the present study, we sought to characterize the pharmacological effects of ethanol on extracellular norepinephrine concentrations in the medial prefrontal cortex. To this end, we utilized two different routes of administration to quantify ethanol's actions. Following an acute intravenous infusion of ethanol, we observed a 77% increase from baseline in dialysate norepinephrine. A control saline infusion did not stimulate extracellular norepinephrine, and in fact we observed a slight decline relative to baseline. In a separate experiment, we performed in vivo microdialysis in rats during an operant self-administration session. Prior to microdialysis, animals had experienced about one week of self-administration of a sweetened ethanol solution or a sucrose solution. To control for the effects of animal handling, a separate group of animals were placed in the operant chambers but did not receive any drinking solutions. We observed a

reduction in basal norepinephrine concentrations in the ethanol-experienced animals relative to control groups. Although there were no significant differences in extracellular norepinephrine activity among the three groups during the operant session, we did observe a transient spike in norepinephrine during the transfer from the home cage to the operant chamber. We conclude that ethanol exerts direct pharmacological actions on central noradrenergic neurons, though the mechanism is unclear. Furthermore, limited voluntary ethanol consumption appears to be sufficient to alter tonic norepinephrine signaling in the medial prefrontal cortex.

INTRODUCTION

Cognitive deficits, such as impairments in working memory, response inhibition, and attention, are well documented in human alcoholics (Bates, Bowden, & Barry, 2002; Potenza, Sofuoglu, Carroll, & Rounsaville, 2011; Stavro, Pelletier, & Potvin, 2013). Such deficits may result from or be exacerbated by drug-induced adaptations in catecholamine signaling in the prefrontal cortex, as suggested by preclinical studies. For example, we recently demonstrated reduced basal dopamine concentrations in the medial prefrontal cortex (mPFC) of rats with about one week of ethanol self-administration experience relative to control animals (Doherty, Schier, Vena, Dilly, & Gonzales, 2016). Although the

functional implications of the reduced mPFC dopamine concentrations were not explored, other work has demonstrated that localized antagonism of dopamine D1-receptors or depletion of dopamine within the prefrontal cortex produces some cognitive deficits (Brozoski, Brown, Rosvold, & Goldman, 1979; Bubser & Schmidt, 1990; Sawaguchi & Goldman-Rakic, 1994). An abundance of literature suggests that cognition is dependent on optimal concentrations of both dopamine and norepinephrine in the prefrontal cortex (Arnsten & Pliszka, 2011; Arnsten, Wang, & Paspalas, 2012; Aston-Jones et al., 1999; Vijayraghavan et al., 2007), and that both neuromodulators may work in concert in the prefrontal cortex to regulate cognition (Sara, 2009; Xing et al., 2016). Pharmacological and lesion studies directly implicate noradrenergic signaling in the mPFC in aspects of cognition, such as sustained attention and attentional set-shifting behaviors (Lapiz & Morilak, 2006; McGaughy, Ross, & Eichenbaum, 2008). Furthermore, drugs such as methylphenidate and atomoxetine are believed to exert their cognitive-enhancing effects by increasing catecholaminergic signaling in the prefrontal cortex (Arnsten & Li, 2005).

The prefrontal cortex receives dopaminergic innervation from the ventral tegmental area and noradrenergic innervation from the locus coeruleus. Within the prefrontal cortex of adult rats, norepinephrine concentrations have been shown to be about 4-5 times higher than dopamine concentrations (Boyce & Finlay, 2009; Slopsema, van der Gugten, & de Bruin, 1982), though another

study has reported that basal dopamine concentrations are slightly higher that norepinephrine concentrations (Pan & Lai, 1995). There is significant overlap in the mechanisms regulating extracellular dopamine and norepinephrine. For example, both neuromodulators are cleared from the synapse primarily via the norepinephrine transporter (NET), which has similar affinity (K_m ~1 micromolar) for both dopamine and norepinephrine (Bymaster et al., 2002; Giros et al., 1994; Morón et al., 2002; Tanda et al., 1997; Valentini, Frau, & Di Chiara, 2004). The relative absence of tyrosine hydroxylase from noradrenergic terminals in the mPFC suggests that norepinephrine in this region may be synthesized by dopamine β-hydroxylase from dopamine taken up by NET (Miner, Schroeter, Blakely, & Sesack, 2003). Furthermore, a recent body of literature indicates that noradrenergic terminals in the prefrontal cortex co-release dopamine and norepinephrine (Devoto, Flore, Pani, & Gessa, 2001; Devoto, Flore, Pira, Diana, & Gessa, 2002; Devoto, Flore, Saba, Fà, & Gessa, 2005).

Ethanol has also been shown to acutely affect performance on cognitive-behavioral tasks in humans and rodents (Popke et al., 2000; Ralevski et al., 2012). Though the underlying mechanisms are unknown, it is possible that ethanol may be altering catecholaminergic activity in the mPFC. We have previously quantified the effects of passive and self-administered ethanol on extracellular dopamine in the mPFC (Doherty et al., 2016; Schier et al., 2013), however, few studies have examined the pharmacological effects of ethanol on

norepinephrine in the mPFC. Ventura and colleagues (2006) demonstrated that selective norepinephrine depletion in the mPFC impairs conditioned place preference for ethanol in mice. An earlier in vivo microdialysis study demonstrated dose-dependent effects of acute ethanol on norepinephrine in the mPFC. A low dose (0.2 g/kg) of ethanol significantly increased norepinephrine, while a high dose (2.0 g/kg) initially increased norepinephrine before significantly declining below baseline (Rossetti, Longu, Mercuro, Hmaidan, & Gessa, 1992). However, because the sampling time in this study was 30 minutes, any transient effects of ethanol on norepinephrine may have been missed. Indeed, in vivo recordings of locus coeruleus neurons demonstrated that although the during a slow intravenous infusion of ethanol showed an initial transient spike followed by a robust decrease in firing rates (Verbanck et al., 1990). Therefore, the first aim of the present work was to quantify the effect of a moderate dose of acute ethanol (1 g/kg) on norepinephrine in the mPFC via in vivo microdialysis with enhanced temporal resolution. Secondly, we sought to quantify extracellular norepinephrine activity in the mPFC during operant self-administration of a sweetened ethanol solution or a sucrose solution. Microdialysis was also performed in a second control group of animals that received access to the operant chambers but did not consume any drinking solutions. Additionally, we used the same experimental design as previously used in our dopamine experiments to determine if about one week of ethanol self-administration would also be sufficient to alter basal cortical noradrenergic signaling. Given the

overlapping mechanisms regulating extracellular concentrations of both dopamine and norepinephrine, we hypothesized that altered basal norepinephrine activity in the mPFC of ethanol exposed animals following seven sessions of operant self-administration.

METHODS

Animals

Final statistical analyses used 27 male adult, Long Evans rats from Envigo Laboratories (Indianapolis, IN, USA; 270-285 g upon arrival). Animals were maintained on a 12-hour light/dark schedule, at 23 ± 2 ºC, with ad libitum food and water (except where noted); rats were weighed each day. All animal procedures complied with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Materials

The intravenous ethanol solution was a 10 % (w/v) solution of ethanol (1 g/kg, 10 ml/kg) in saline was made from 95 % ethanol (Aaper Alcohol and

Chemical Co., Shelbyville, KY). Drinking solutions (10S: 10% sucrose (w/v) or 10S10E: 10% ethanol (v/v) in 10S) were made from 95% ethanol (AAPER Alcohol and Chemical Co., Shelbyville, KY), ultra-pure sucrose (Fisher Scientific, Pittshburgh, PA) and tap water. Carprofen (Pfizer, New York, NY) and gentamicin (APP Pharmaceuticals, Schaumburg, IL) were used during surgery.

Surgery

Cannulation and jugular catheterization surgeries were carried out according to the procedures described in Duvauchelle (1998) and Howard et al. (2008). Intravenous catheters were constructed from silastic tubing (0.30 mm ID, 0.64 mm OD, Fisher Scientific, Hampton, NH), a metal cannula (22 gauge, Plastics One, Roanoke, VA), and silicone adhesive. Rats were anesthetized with isoflurane and an incision was made above the skull. Upon securing the catheter in the jugular vein, it was pulled subcutaneously to the top of the skull. A microdialysis guide cannula (21 gauge, Plastics One, Roanoke, VA) was implanted into the skull directly above the medial prefrontal cortex (AP +3.0, ML +0.57, DV -1.1) while the animal was in a stereotaxic frame. The guide cannula, catheter cannula, and a tether bolt were held in place on the skull with dental cement. Catheters were flushed with 0.2 mL of heparinized saline at least once a week before dialysis experiments commenced. For the self-administration

experiments, rats were not catheterized and only underwent intracranial cannulation surgery. Following surgeries, rats were individually housed and given at least 6 days of recovery prior to experiments.

Microdialysis

Approximately 12-18 hours prior to the microdialysis experiment, rats were lightly anesthetized with isoflurane to implant the microdialysis probe through the guide cannula and to secure the animal to the tethering apparatus. The probes (3 mm active membrane length, 270 µm OD, 13,000 MWCO) were constructed in the laboratory according to the procedures described by Pettit & Justice (1991). Probes were continuously perfused with artificial cerebrospinal fluid (ACSF; 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 5.4 mM D-glucose) overnight at 0.2 µL/min. The flow rates were increased to 1.0 µL/min at least 2 hours prior to dialysate sample collection and remained at 1.0 µL/min for the duration of the experiment.

Animals were awake and freely moving during the microdialysis experiments. In all experiments, the sample collection interval was 10 minutes and three baseline samples were collected per animal in the home cage prior to any experimental manipulations. Baseline norepinephrine concentrations were required to have a relative standard deviation <0.30 for data inclusion. To confirm

that the norepinephrine in the dialysate samples was due to calcium-dependent exocytotic release, probes were perfused with calcium-free ACSF for approximately 2 hours at the conclusion of all experiments and additional samples were collected. All dialysate samples were immediately frozen on dry ice upon collection.

Self-Administration Training and Protocols

Groups

Rats were initially trained to lever press for access to a 10% sucrose (10S) solution, and then two groups were formed, one that consumed 10% ethanol in 10% sucrose (10S10E) and a group that continued to drink 10S. The Handling control group was exposed to all the same procedures as the 10S10E and 10S groups (physical handling, water deprivation during lever press training, time in the operant chamber, tethering, and dialysis), but they were not exposed to drinking solutions or operant training.

Lever-press training and operant protocol

At least 6 days after surgery, animals were habituated to operant chambers (Med Associates, Inc., Vermont, USA) and then trained to lever press for a 10S solution (10S10E and 10S groups only). Water deprivation (maximum 22 hours/day) was used to expedite lever-press training. Animals typically learned to lever press within 1-2 training sessions (one session/day), after which they regained ad libitum access to water for the remainder of the experiment. Operant chambers were as previously described by Howard et al. (2009). Briefly, chambers had a retractable lever, sipper tube bottle, house light, cue light and lickometer circuit, and were contained in sound-attenuating boxes with the doors removed to accommodate microdialysis sample collection. Med Associates software was used to run the operant programs.

Once trained to lever press, animals began an eight-session training schedule during which a pre-lever-press wait period was lengthened from 0-30 min, and the response requirement was increased from 2-4. Following completion of the response requirement, the sipper tube containing the drinking solution entered the chamber for 30 minutes, during which animals had ad libitum access to the drinking solution. No further responding was required. Sessions were run once a day, four to six days per week. Animals received a total of three to four, but never more than two sequential days off from training once the eight-session protocol began. All sessions were run between the hours of 9:00 am – 12:00 pm, during the animal's light cycle. The room light was turned off during all

sessions to minimize distractions. The handling control group completed the same procedures, except drinking solutions were not available, and the lever was present but pressing had no consequences. Drinking bottles were weighed before and after the drinking session to quantify solution consumption, and pattern of consumption was monitored using the lickometer. Any spillage or evaporation was accounted for in the final consumption calculations.

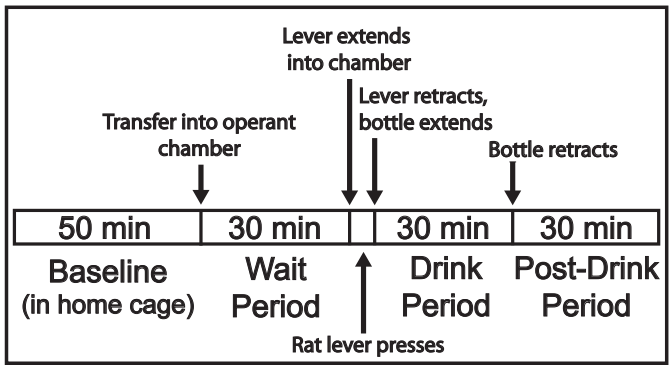
Following the sixth operant session, a spring was attached to the tether bolt on the animal's head and connected to a swivel suspended above the rat by a counter-balance lever arm. Rats were tethered in their home cages (placed next to their operant chamber) overnight and during the seventh operant session to facilitate habituation to the apparatus. The tethering apparatus did not interfere with the rats' abilities to move freely about their home cage or to lever press in the operant chamber. Following completion of the seventh operant session, animals underwent the microdialysis procedure as described above.

Microdialysis Timeline

Microdialysis samples were manually collected every 10 minutes before and during the eighth operant session (Figure 19). Three baseline samples were taken in the home cage. During the last minute of the fourth sample, the rat was transferred into the operant chamber. The operant program began with turning

on the house light and sound-attenuating fan, and the sample collection vial was changed to the first wait period sample. Three wait period samples were taken. The time it took the rat to meet the response requirement of four lever presses was collapsed into the last wait period sample. The wait/lever-press sample was changed to the first drink sample as the sipper tube entered the chamber. Three samples were taken during the drink period, after which the bottle retracted and the house light turned off. Then three samples were taken during the post-drink period. The rat was then returned to its home cage, and the ACSF was changed to calcium-free ACSF. For the 10S10E group, two microliter aliquots were removed from the sample before the lever extended into the chamber and all subsequent samples to determine dialysate ethanol content. Animals receiving 10S10E were required to consume at least 0.8 g/kg during the microdialysis operant session for inclusion in the final analyses.

Figure 19: Experimental timeline for operant self-administration session on the 8th day of testing.



Microdialysis was performed during the final operant session, and consisted of 4 phases during which dialysate samples were collected every 10 minutes. Figure adapted and modified from Doherty et al, 2016

HPLC Analysis

Dialysate norepinephrine concentrations were quantified via reversed-phase high performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a Waters Acquity UPLC column (either HSS T3 1.0 x 50 mm or BEH, 1.0 x 100 mm; C18, 1.7 µm particle size; Waters Corporation, Milford, MA), 2 mm glassy carbon working electrode electrochemical detector (SenCell; Antec Leyden) at potential +450 mV, an 8125 manual injector (Rheodyne, Cotati, CA), and a Decade ELITE controller (Antec

Leyden). Mobile phase was continuously pumped through the system via an LC110S pump (Antec Leyden). The mobile phase consisted of 0.500 g octanesulfonic acid, 0.150 g decanesulfonic acid, 0.128 g ethylenediaminetetraacetic acid, and 11.08 g NaH_2PO_4 dissolved in 1 liter of deionized water, and methanol as the organic solvent (4-10% v/v). The mobile phase was adjusted to pH 5.6 prior to adding the methanol. The sample injection volume was 5 microliters. External standards (0.16 to 2.5 nM) were used to quantify the norepinephrine concentrations. EZChrom Elite software (Agilent, Wilmington, DE) was used to record and analyze all chromatograms. Only norepinephrine peaks with a signal to noise ratio >6 were included in the analyses.

GC Analysis

For animals that received ethanol infusions, dialysate ethanol concentrations were quantified via gas chromatography (GC) with flame ionization detection (Robinson, Lara, Brunner, & Gonzales, 2000). Prior to freezing the dialysate samples, 2 µL aliquots were transferred to 2 mL glass chromatography vials and sealed with a septum. The GC system consisted of a Scion 436 gas chromatograph (Bruker, Netherlands), a Varian 8200 headspace autosampler, and hydrogen (via a hydrogen generator; Model 20H-MD, Parker

Hannifin, England) as the carrier gas. The stationary phase was an HP Innowax capillary column (30 m × 0.53 mm × 1.0 μm film thickness). Resulting ethanol peaks were recorded using CompassCDS (Bruker, Netherlands) software, and calibration was achieved using external standards (0.3125 to 40 mM). For one animal in the operant self-administration experiment, some samples were lost due to a technical malfunction.

Histological Analysis

Within three days of dialysis, animals were overdosed with sodium pentobarbital (150 mg/kg, intraperitoneal). Brains were extracted and placed in jars containing 10% formalin in saline. At least 48 hours later, brains were coronally sectioned (120 μm thick), and stained with cresyl violet for verification of the microdialysis probe placement (Paxinos et al., 2007).

Statistical Analyses

Normalized norepinephrine concentrations (represented as a percent of baseline) were analyzed using repeated measures analysis of variance (ANOVA) in both the acute and self-administration experiments. Post hoc analyses (using

pooled error and Bonferroni corrections, as appropriate) followed up on any significant findings from the overall ANOVA.

The acute intravenous experiments followed a within subjects design with animals receiving both a saline and an ethanol infusion. The within subjects factor was time, and separate ANOVAs were performed on the saline and ethanol data.

In the operant self-administration experiments, treatment was the between-subjects factor (three levels: 10S10E, 10S, and handling) and time (13 time points: 3 home cage baseline, 4 wait period, 6 drink and post-drink) as within subjects repeated factor. Specifically, when significant interactions between main effects were observed in the omnibus ANOVA, simple effects analyses were done to determine the source of the significant interaction.

RESULTS

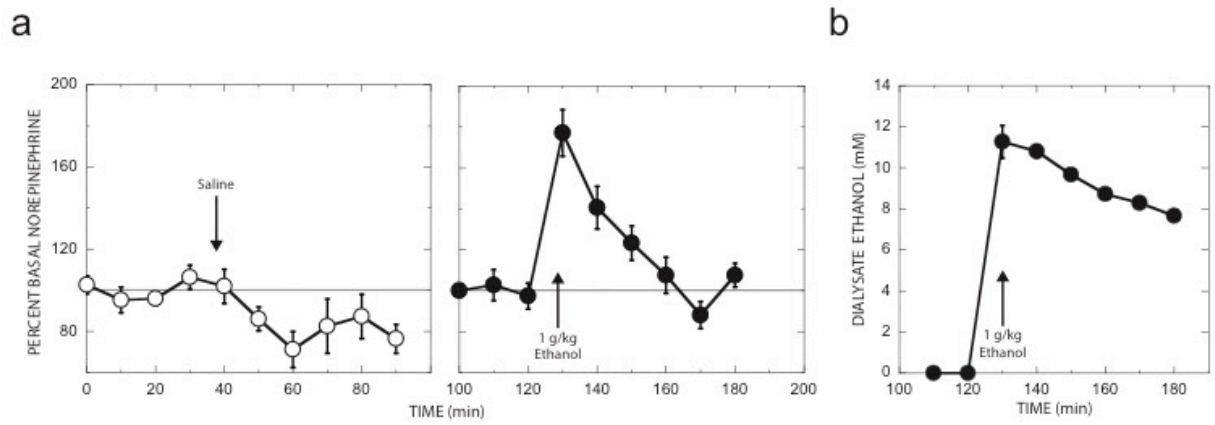
Acute systemic ethanol (1 g/kg) stimulates extracellular norepinephrine in the medial prefrontal cortex

The mean dialysate norepinephrine concentration in the basal samples prior to the saline infusion was 0.32 ± 0.04 nM. Prior to the ethanol infusion, the mean dialysate norepinephrine concentration was 0.24 ± 0.01 nM.

Separate repeated measures ANOVAs were performed on the pre- and post-saline infusion samples and the pre- and post-ethanol samples. Analyzing the normalized data, the overall ANOVA on the baseline and post-saline infusion samples revealed a simple effect of time (Figure 20a, left panel; $F_{9,54}$=2.73, p=0.01). Post hoc analysis indicated significance only at the 60-minute time point ($F_{4,54}$=3.71, p<0.01; 20 minutes following the saline infusion). The overall ANOVA on the pre- and post-ethanol infusion samples also revealed a simple effect of time (Figure 20a, right panel; $F_{8,48}$=12.80, p<0.01). The ethanol infusion significantly increased extracellular norepinephrine to 77% above baseline in the dialysate sample immediately following the ethanol infusion. Post hoc analysis indicated significance at the 130-minute ($F_{3,48}$=25.05, p<0.01) and the 140-minute ($F_{3,48}$=6.98, p<0.01) time points.

The dialysate ethanol concentrations from all samples subsequent to the infusion are depicted in Figure 20b. Dialysate ethanol concentrations peaked at 11.3 ± 0.8 mM immediately following the infusion and steadily declined throughout the microdialysis experiment.

Figure 20: Extracellular norepinephrine (normalized to baseline) in the mPFC following intravenous administration of saline and 1 g/kg ethanol.



(a) Extracellular norepinephrine (normalized to baseline) in the medial prefrontal cortex following intravenous administration of saline (left panel) and 1 g/kg ethanol (right panel). (b) Dialysate ethanol concentrations following intravenous administration of 1 g/kg ethanol. The arrows indicate the time of the infusions. Data points represent mean ± SEM; significance from baseline is indicated by asterisks (*).

Ethanol consumption during operant self-administration sessions

The ethanol consumption data for the 10S10E group is shown in Table 6. Over the four sessions prior to microdialysis, animals consumed on average 1.4 ± 0.2 g/kg. During the microdialysis session, animals consumed 1.6 ± 0.2 g/kg.

Basal norepinephrine concentrations in the medial prefrontal cortex following limited voluntary ethanol consumption

Microdialysis was performed in three different groups of animals following about one week of operant self-administration. The baseline dialysate norepinephrine concentrations for the handling and 10S control groups were 0.50 ± 0.04 nM and 0.57 ± 0.04 nM, respectively (Figure 21). The baseline dialysate norepinephrine concentrations for the 10S10E group were 0.33 ± 0.04 nM, significantly lower than those of the control groups ($F_{2,14}$=7.02, $p<0.01$).

Table 6: Ethanol consumption during operant self-administration sessions

Session	Ethanol consumption (g/kg)
1	N/A
2	0.30 ± 0.03
3	1.08 ± 0.21
4	1.75 ± 0.30
5	1.27 ± 0.17
6	1.46 ± 0.17
7	1.26 ± 0.18
8 (Microdialysis)	1.58 ± 0.17

Data are mean ± SEM

Dialysate norepinephrine during operant self-administration

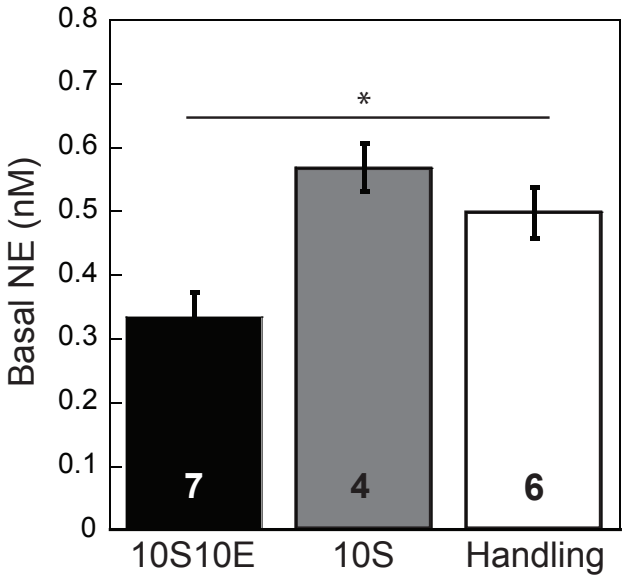
The operant microdialysis session consisted of 3 phases: home cage baseline, transfer and wait period, and drink/post-drink period. Although an overall ANOVA on the normalized data revealed a significant effect of time (Figure 22; $F_{12,168}$=8.78, $p<0.01$), there was no time by treatment interaction ($F_{24,168}$=0.81, NS) and no main effect of treatment ($F_{2,14}$=0.18, NS).

Post hoc analyses indicated that the main effect of time was driven by a significant increase in extracellular norepinephrine at the time of transfer from the home cage into the operant chamber, which occurred in all 3 groups. Significant increases from baseline were observed at the 40-minute ($F_{3,168}$=30.45, $p<0.001$; collapsed across treatment groups) and 50-minute ($F_{3,168}$=9.07, $p<0.001$; collapsed across treatment groups) time points.

Dialysate ethanol concentrations during final operant session

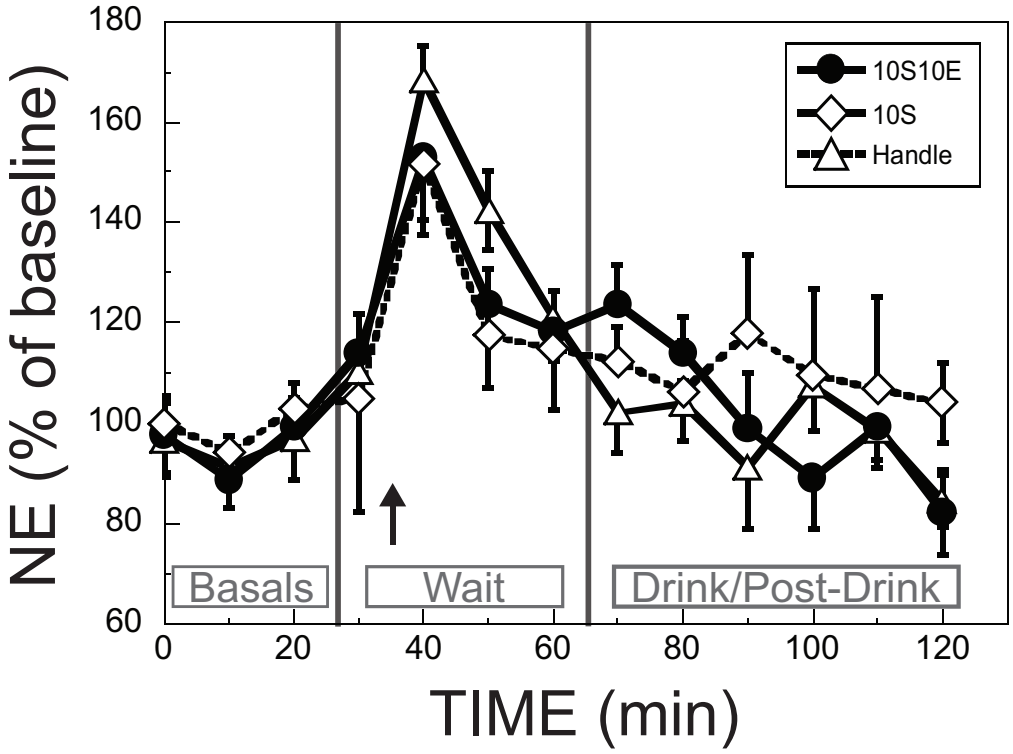
For the animals in the 10S10E group, dialysate ethanol concentrations were determined via gas chromatography (Figure 23). Dialysate ethanol concentrations rose over the course of the drink/post-drink period, peaking at 6.1 ± 1.4 mM at 40 minutes following the start of the drink period.

Figure 21 Basal norepinephrine concentrations in the medial prefrontal cortex following limited voluntary operant self-administration.



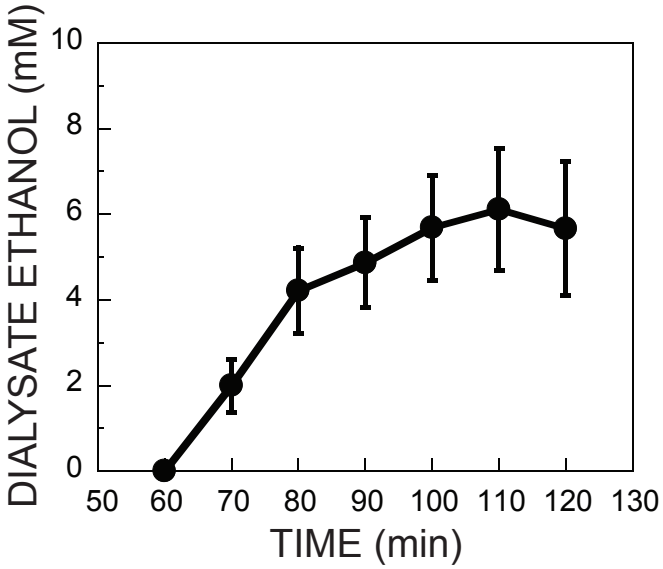
Animals received a sweetened ethanol solution (10S10E), a sucrose solution (10S), or nothing (Handle) over seven operant sessions prior to microdialysis. Basal samples were collected over 30 minutes while the animals remained in their home cages. Data points represent mean ± SEM; significant group differences indicated by the asterisk (*). The n per group is indicated by the numbers within each bar.

Figure 22: Dialysate norepinephrine (normalized to baseline) during the 8th operant self-administration session.



During the final operant session, basal samples were collected prior to any manipulations. The animals were physically transferred (indicated by the arrow) to the operant chambers, initiating the onset of the 30-minute wait period. Upon completion of the response requirement, animals received 30 minutes of access to a sweetened ethanol solution (10S10E), a sucrose solution (10S), or no reinforce (Handle). Data points represent mean ± SEM.

Figure 23: Dialysate ethanol concentrations during final operant session.



Data points represent mean ± SEM.

DISCUSSION

The current work demonstrates that intravenous administration of an intoxicating dose of ethanol acutely stimulates extracellular norepinephrine in the medial prefrontal cortex (mPFC) of naïve animals. While we observed a decline in extracellular norepinephrine following the saline infusion, this may have been due to the behavioral state of the animals. Typically, animals fall asleep or lay down after about 30 minutes into the acute microdialysis experiment, and norepinephrine levels are known to decrease during low arousal states (España, Schmeichel, & Berridge, 2016; Feenstra, 2000).

Additionally, this is the first study to quantify norepinephrine activity in the mPFC during an operant self-administration session. We monitored extracellular norepinephrine activity in animals that had consumed a sweetened ethanol solution (10S10E), a 10% sucrose solution (10S), or nothing (handling) in the operant chamber for about one week prior to the microdialysis experiment. In all three groups, we observed a significant increase in extracellular cortical norepinephrine relative to baseline at the time of transfer into the operant chamber. Although this is consistent with other reports of stimulated norepinephrine concentrations in the mPFC during physical handling (Feenstra, Botterblom, & Mastenbroek, 2000; Ihalainen, Riekkinen Jr, & Feenstra, 1999; Kawahara, Kawahara, & Westerink, 2000; Marsteller et al., 2002), we cannot rule out the influence of appetitive stimuli on cortical norepinephrine activity. A

potential confound of these earlier studies is that the handling period lasted 10-16 minutes, which may be stressful. In contrast, when the handling period is transient, such as during the transfer from one cage to another, there is no apparent effect on extracellular cortical norepinephrine (Rossetti & Carboni, 2005). Additionally, conditioned appetitive and aversive stimuli have been shown to stimulate extracellular norepinephrine in the mPFC (Mingote, de Bruin, & Feenstra, 2004; Ventura, Latagliata, Morrone, La Mela, & Puglisi-Allegra, 2008). Therefore, it is possible that the noradrenergic response observed during the transfer period may be attributed to the conditioned stimuli associated with the operant chambers, but further experiments are necessary to confirm this hypothesis.

Although we did not observe differences in norepinephrine activity among the three groups of animals, we did observe significant differences in tonic norepinephrine, with the 10S10E group showing reduced basal levels relative to the 10S and handling groups. Interestingly, we have previously reported a reduction in dopaminergic tone in the mPFC of ethanol-experienced animals relative to control animals under the same experimental conditions (Doherty et al., 2016). Together, our work is the first to demonstrate altered catecholaminergic signaling in the mPFC following limited voluntary ethanol self-administration. Our observations are consistent with an early study that measured tissue concentrations of dopamine and norepinephrine in two strains

of mice following 7-10 days of a forced liquid ethanol diet. Both strains of mice demonstrated a reduction in basal levels of both catecholamines in the cortex (Shafik, Aiken, & McArdle, 1991). Additionally, another study in rats found reduced tissue concentrations of norepinephrine in the mPFC in rats given two weeks of a liquid ethanol diet, relative to pair-fed controls (Carlson & Drew Stevens, 2006). However, in these studies, it is unclear if extracellular concentrations were specifically reduced. Our interpretation of these findings is limited as minimal work exists investigating ethanol's interactions with the mechanisms regulating extracellular concentrations of cortical catecholamines. Therefore, at this time, we can only speculate as to the mechanisms underlying our reports of reduced catecholaminergic tone in animals with limited voluntary ethanol consumption experience relative to ethanol-naïve controls.

The literature contains an abundance of work exploring the effects of stress on catecholaminergic regulation in the prefrontal cortex that may have some relevance to the current work. Ethanol itself has been shown to activate the endogenous stress system (Lee & Rivier, 1997; Soon Lee, Selvage, Hansen, & Rivier, 2004; Rivier, 1996). Similar to ethanol, acute stress also stimulates extracellular dopamine and norepinephrine in the mPFC (Finlay, Zigmond, & Abercrombie, 1995; Nakane et al., 1994). Additionally, following chronic exposure to a severe stressor for 4 weeks, rats showed a reduction in basal dopamine concentrations in the mPFC relative to unstressed control rats

(Mizoguchi et al., 2000). A separate study demonstrated an increase in membrane-bound NET in the mPFC following 2 weeks of chronic cold stress (Miner et al., 2006), which theoretically would increase dopamine and norepinephrine clearance. However, the same stress paradigm produced no significant differences in basal norepinephrine concentrations between stress and unstressed animals (Gresch, Sved, Zigmond, & Finlay, 1994; Jedema, Sved, Zigmond, & Finlay, 1999). While it is unknown if chronic ethanol administration alters prefrontal catecholamine signaling via the same mechanism as chronic stress, our reports of reduced basal catecholamines could be explained by an enhancement of NET activity.

Alternatively, ethanol may be interacting with catecholamine metabolic enzymes, another mechanism of clearance in the prefrontal cortex. Following administration of a high dose of ethanol (3.5 g/kg, i.p.), dopamine metabolite concentrations were significantly elevated in the PFC of mice, relative to controls. Increased concentrations of a norepinephrine metabolite were also observed, but this effect was not statistically significant (Milio & Hadfield, 1992). In humans, elevated concentrations of a norepinephrine metabolite have been observed in the CSF of alcoholics and healthy controls following acute administration of an intoxicating dose of ethanol (Borg, Kvande, Sedvall, & Goldman, 1981). However, in these studies, it is unclear if the increased concentrations of catecholamine metabolites is the result of ethanol-induced alterations in the

function of metabolic enzymes, or if they are simply the result of stimulated dopamine and norepinephrine concentrations following acute ethanol administration. Lastly, ethanol may be inducing adaptations in the mechanisms regulating catecholamine synthesis and release, such as autoreceptor feedback control mechanisms. Further investigation is required to explore these hypotheses.

Several studies have examined the effect of pharmacologically targeting the noradrenergic system on alcohol self-administration in humans and rodents. In general, these studies report that drugs that have a net effect of activating the noradrenergic system, appear to enhance alcohol seeking and consumption. In contrast, drugs that reduce noradrenergic signaling appear to reduce alcohol seeking and consumption. For example, systemic prazosin, an a1-receptor antagonist, reduced ethanol-seeking behavior in non-dependent animals, but only at the highest tested dose (Walker, Rasmussen, Raskind, & Koob, 2008). Prazosin also reduced ethanol consumption and seeking behavior in alcohol-preferring P rats (Verplaetse, Rasmussen, Froehlich, & Czachowski, 2012). Human alcohol dependent patients treated with prazosin also reported fewer drinking days and fewer drinks per week than those receiving placebo (Simpson et al., 2009). Similar effects have also been observed with another a1-receptor antagonist, doxazosin (Kenna et al., 2016; O'Neil, Beckwith, Kincaid, & Rasmussen, 2013), and with clonidine (Rasmussen, Alexander, Malone,

Federoff, & Froehlich, 2014) and guanfacine (Riga, Schmitz, et al., 2014), both of which reduce noradrenergic signaling by agonizing a2-receptors. Together, these studies appear difficult to reconcile with our observation of reduced noradrenergic tone in the mPFC following about one week of alcohol self-administration. However, it is necessary to consider the clinical or preclinical populations in which these noradrenergic agents were tested.

In the current study, the 10S10E animals were unlikely to experience significant tolerance and withdrawal due to the limited self-administration experience. In contrast, the clinical studies in which a1 antagonism reduced drinking were conducted in alcohol-dependent individuals. Furthermore, in the studies conducted by Rasmussen et al (2014) and Riga et al (2014), animals had prolonged (at least 9 weeks) ethanol self-administration experience prior to being treated with clonidine and guanfacine, respectively. The central noradrenergic system may be differentially regulated in the early stages of alcohol use and following prolonged alcohol use. With chronic and prolonged alcohol use, withdrawal symptoms emerge that are associated with activation of the sympathetic nervous system, in which norepinephrine is a key mediator. For example, a hyperexcitability endophenotype has been observed in alcohol-dependent males (Krystal et al., 1997), alcoholics undergoing acute withdrawal (Begleiter & Porjesz, 1999), alcohol-preferring P and HAD rats (Chester, Blose, & Froehlich, 2004), and ethanol-experienced mice undergoing acute withdrawal

(Kovács, Soroncz, & Tegyei, 2002). This endophenotype is associated with greater sensitivity to the stimulatory effects experienced during the ascending limb of the blood alcohol curve (Begleiter & Porjesz 1999) and enhanced noradrenergic activation (Kovács et al., 2002). Studies in humans and animals report either normal or reduced peripheral noradrenergic signaling in dependent subjects not experiencing withdrawal symptoms relative to controls. However, with the onset of ethanol withdrawal, there is an abrupt upregulation of the peripheral noradrenergic system (Fitzgerald, 2013; Kovács et al., 2002; Mäki et al., 1990; Patkar et al., 2003; Smith, Brent, Henry, & Foy, 1990). Furthermore, plasma norepinephrine concentrations are positively correlated with withdrawal scores (Kovács et al., 2002; Smith et al., 1990). Therefore, following voluntary alcohol self-administration in non-dependent subjects, there may be an initial reduction in noradrenergic activity, but following repeated withdrawal experiences there may be an upregulation of noradrenergic signaling, as suggested by elevations in norepinephrine metabolites in the CSF and plasma of alcoholics undergoing withdrawal (Linnoila, Mefford, Nutt, & Adinoff, 1987; Nutt, Glue, Molyneux, & Clark, 1988). Additionally, plasma concentrations of a norepinephrine metabolites correlated with a history of alcohol withdrawal-induced hospitalizations and the number of years of experiencing withdrawal symptoms (Linnoila et al, 1987). These findings and others provide evidence for a sensitized sympathetic nervous system following multiple alcohol withdrawal episodes, potentially mediated by an upregulated noradrenergic system.

However, there is remarkably limited work monitoring central norepinephrine concentrations during alcohol withdrawal.

Lastly, reduced norepinephrine signaling could have effects on cortical glial activity. Several recent studies have suggested that locus coeruleus norepinephrine neurons exert neuromodulatory effects on astrocytes that may promote widespread coordination of neural-glial networks (Fuxe, Agnati, Marcoli, & Borroto-Escuela, 2015). For example, in an aroused behavioral state or following stimulation of locus coeruleus neurons, enhancement of astrocytic Ca^{2+} signaling throughout the cortex has been reported (Bekar, He, & Nedergaard, 2008; Ding et al., 2013; Paukert et al., 2014). Additionally, alpha-1 and beta-1/beta-2 adrenergic receptors have been identified on cortical astrocytes, and norepinephrine (as well as α1 and β1/ β2 agonists) activates astrocytes via these receptors (Ding et al., 2013; Fuxe et al., 2015; Laureys et al., 2010). Activation of astrocytic adrenergic receptors has been shown to be neuroprotective and facilitates anti-inflammatory signaling. For example, application of norepinephrine to rat cortical slices or cell cultures increased levels of brain-derived neurotrophic factor (BDNF) and MCP-1 in astrocytes, which function as neuroprotective factors when released by astrocytes (Jurič, Lončar, & Čarman-Kržan, 2008; Laureys et al., 2010; Madrigal, Leza, Polak, Kalinin, & Feinstein, 2009). Furthermore, activation of astrocytic β2 adrenergic receptors blocks the synthesis of nuclear factor kappa B (Nf-κB) derived pro-inflammatory signaling molecules,

such as TNF-α and IL-β, and upregulates the anti-inflammatory pathway mediated by peroxisome proliferator-activated receptor gamma (PPAR-Y) (Fuxe et al., 2015; Laureys et al., 2010). Therefore, a possible consequence of reduced prefrontal cortical norepinephrine signaling may be altered regulation of astrocyte-mediated immune activity, potentially resulting in increased pro-inflammatory signaling. However, because norepinephrine has a lower affinity for α1 and, more so, for β adrenergic receptors, it is unclear if the reduction in basal norepinephrine concentrations observed in the current study is significant enough to influence astrocytic activity.

In summary, we have quantified the direct pharmacological effects of acute ethanol on extracellular norepinephrine activity in the mPFC. Furthermore, the current work provides evidence of ethanol-induced alterations in basal noradrenergic signaling in the mPFC, a region known to exert top-down behavioral control. Previous work found similar effects on basal dopamine concentrations in the mPFC using the same self-administration paradigm. These observations may be the result of ethanol's complex mechanism of action; ethanol may be interacting with catecholaminergic synthesis, release, and/or clearance mechanisms to produce alterations in basal cortical concentrations. Functionally, a deficit in tonic catecholamine signaling in the mPFC could impair cognitive and executive processes similar to the impairments observed in ADHD and PTSD, such as poor inhibitory control, deficits in behavioral flexibility, and reductions in working memory (Arnsten & Li, 2005; Arnsten & Pliszka, 2011;

George et al., 2015; Swick, Honzel, Larsen, & Ashley, 2013). The functional consequences of reduced catecholamine signaling in the mPFC were not explored in the current work, however, so additional studies are necessary to determine if the observed reduction in basal catecholamine concentrations are sufficient to alter behavior.

Chapter 6:

Concluding remarks and future directions

SUMMARY OF FINDINGS

The work presented within this dissertation provides critical information on the differential actions of ethanol on catecholamine activity in the medial prefrontal cortex and striatal subregions. Defining ethanol's precise mechanism of action has been highly elusive for several reasons. As discussed in Chapter 2, ethanol's chemical properties enable it to interact with various substrates involved in highly diverse physiological functions. Even ethanol's actions on midbrain dopamine circuits are highly heterogeneous, and alone do not explain precisely how ethanol acts to contribute to the development of alcohol use disorders. This is evident in Chapter 3, where we quantitate the differential pharmacological effects of acute intravenous ethanol on extracellular dopamine in the nucleus accumbens, dorsomedial striatum, and dorsolateral striatum. Our findings indicate a potential, albeit limited role of dorsomedial striatal dopamine in the acute reinforcing effects of ethanol, and this may contribute to the development of ethanol seeking behaviors by facilitating the learning of action-outcome contingencies (Yin et al., 2008).

While these initial studies used intravenous administration to quantify ethanol's acute pharmacological effects on extracellular dopamine, a self-

administration model possesses enhanced face validity for human alcohol use. Our analyses of the dopamine-ethanol “response ratios” in Chapter 2 suggests that ethanol exerts particularly unique effects on the regulation of extracellular dopamine in the medial prefrontal cortex (mPFC). Consistent with this, we are the first group to report reductions in basal dopamine in the mPFC in non-dependent animals with limited ethanol self-administration experience, relative to controls (Chapter 4).

Because ethanol’s effects on neurobiology, neurochemistry, and behavior extend beyond its interactions with a heterogeneous population of midbrain dopamine neurons, we also examined the effects of ethanol on cortical noradrenergic activity. In Chapter 5, we demonstrate that acute intravenous ethanol robustly stimulates extracellular norepinephrine in the mPFC. Similar to what we have reported with dopamine, we also observed a dissociation in the temporal profiles of dialysate ethanol and norepinephrine following acute administration. After about one week of self-administration of a sweetened ethanol solution, however, systemic ethanol does not alter norepinephrine activity in the mPFC. Whether this observation indicates tolerance to the stimulatory effects of ethanol or is due to differences in the route of administration remains to be investigated. Intriguingly, the same study also reported a reduction in basal norepinephrine concentrations in the mPFC of animals consuming sweetened ethanol for about one week, relative to sucrose and handling controls. Together, the findings of Chapters 4 and 5 demonstrate that even with voluntary, goal-

directed ethanol consumption, there are alterations in the mechanisms regulating extracellular catecholamine activity in the mPFC.

IMPLICATIONS AND FUTURE DIRECTIONS

The findings presented here incrementally contribute to our understanding of the mechanisms by which ethanol targets central catecholamine signaling. These studies also invite several opportunities for future investigation. For example, as discussed in Chapter 2, the temporal dissociation in dialysate dopamine and ethanol concentrations following acute ethanol administration may have functional relevance, which remains to be tested. Acute functional tolerance to the stimulatory or sedative effects of ethanol represents a rapid neuroadaptation that occurs on the time scale of minutes and is dose-dependent. Ponomarev and colleagues determined that acute functional tolerance in mice approaches its maximum for a given dose within 10-20 minutes following an injection of ethanol (Ponomarev & Crabbe, 2004). Although it is important to consider differences in ethanol metabolism between species, this time frame may match the onset of the temporal dissociation between dialysate dopamine and ethanol concentrations in the nucleus accumbens that we have observed in rats. However, microdialysis studies lack the temporal resolution to confirm this. Future studies using techniques with enhanced temporal resolution to monitor midbrain dopamine neuron activity can assess whether specific aspects of acute

functional tolerance are associated with rapid adaptations in the sensitivity of mesolimbic dopamine neurons to acute ethanol.

Additionally, voluntary ethanol self-administration appears to alter the mechanisms regulating extracellular catecholamine concentrations specifically in the mPFC, but the mechanisms are unknown. Quantitative microdialysis can be employed to confirm the observed ethanol-induced reduction in basal catecholamine concentrations in the mPFC. Additionally, quantitative microdialysis could help determine if ethanol is modulating release or clearance mechanisms to produce this effect. While several studies have examined ethanol's interactions with the mechanisms regulating extracellular dopamine, little is known about the effects of ethanol on the mechanisms regulating extracellular norepinephrine.

Additionally, the question remains whether the observed ethanol-induced reduction in basal catecholamine concentrations in the mPFC is functionally relevant. As discussed in Chapter 1, proper mPFC function relies on optimal concentrations of dopamine and norepinephrine, and the mPFC is highly sensitive to neurochemical changes (Robbins & Arnsten, 2009). Therefore, it is reasonable to hypothesize that the observed reduction in basal catecholamine concentrations in the mPFC of ethanol-experienced animals is sufficient to induce behavioral deficits. Reduced catecholamine activity in the mPFC is associated with impairments in inhibitory control, behavioral flexibility, working memory and attentional selection (Dalley, Theobald, Eagle, Passetti, & Robbins,

2002; Robbins & Arnsten, 2009; Rossetti & Carboni, 2005). Such impairments have been associated with increased alcohol consumption (Bogg, Fukunaga, Finn, & Brown, 2012) and are prominent in human alcoholics (Noël et al., 2001; Xavier Noël et al., 2005). Indeed, reduced dopamine transmission in the mPFC has been reported in human alcoholics (Narendran et al., 2014) and in rats exposed to a chronic intermittent ethanol protocol, which also displayed deficits in behavioral flexibility (Trantham-Davidson et al., 2014). Additionally, recent human imaging studies have demonstrated regional reductions in white and grey matter in the mPFC in individuals with mild (Asensio et al., 2016) and severe (Wang et al., 2016) alcohol use disorders, and these structural abnormalities were associated with increased impulsivity. Together, our work in conjunction with existing work indicates that ethanol-induced reductions in basal catecholamine concentrations in the mPFC may facilitate the development of impaired control over alcohol use. However, additional work is necessary to determine if the reduction in extracellular catecholamines that we observed is sufficient to produce deficits in behavioral flexibility or impulse control.

Lastly, this work is consistent with earlier studies implicating mPFC dopamine in alcohol self-administration (Hodge et al., 1996; Samson & Chappell, 2001), but it also provides novel data indicating a potential role of mPFC norepinephrine in maintaining alcohol self-administration. However, additional studies are necessary to determine if the observed ethanol-induced alterations in catecholamine signaling in the mPFC can be reversed with abstinence, and it if

has any bearing on the activity of mPFC neuronal projection targets, such as striatal subregions, ventral tegmental area, and the locus coeruleus. Furthermore, the findings presented here provide clear evidence for ethanol's acute actions on the central noradrenergic system. While many previous studies have focused on norepinephrine's role in alcohol withdrawal, we have demonstrated adaptations in cortical norepinephrine signaling following only about one week of ethanol self-administration experience in non-dependent animals.

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Vita

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